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THE INFLUENCE OF SENESCENCE ON THYROID FUNCTION: FUNCTIONAL CHANGES EVALUATED WITH I^{131}

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THE functional status of the thyroid gland in old age and its role in the genesis of some of the manifestations of senescence have been subjects of speculation for many years. It has been recognized that some of the features of senescence are similar to those of the hypothyroid state: thus dryness of the skin, diminished motor activity, impaired muscle strength, diminished cold tolerance and sparseness of the hair are common to both. Lorand (1) commented on the similarity of ageing to hypothyroidism and others (2) have suggested thyroid hormone therapy for the aged. Carlson (3), in a recent review of thyroid function during ageing, states that there is no convincing proof that secretion of thyroid hormone decreases with advancing age. In a previous publication from this laboratory (4) thyroid secretion rate, determined by the goiter prevention assay, was less in senescent rats than in younger mature animals. This observation has been extended by the studies herein reported.

MATERIALS AND METHODS

Female Wistar rats obtained from Manor Farm were used throughout. The young rats were 4–5 months old and the senescent animals 24–25 months. They were housed in individual cages at $26 \pm 1°$ C and at 45–55% humidity. The atmospheric pressure in the room varied from 74–78 cm. Hg. and the lights were controlled to go on at 8:00 A.M. and off at 8:00 P.M. The animals were placed on a Remington iodine deficient diet (5) for 10 days to increase the uptake of I^{131} and to eliminate the influence of small amounts of thyroxine that might be present in the ordinary diet (6).

In Vivo Studies: A modification of the method of Fish *et al.* (7) was used for the *in*

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vivo measurement of thyroid radio-iodine. A G10Pb Geiger counter tube was enclosed in a 1" lead shield with a $\frac{3}{4}$ aperture. The necks of the animals were shaven and the larynx located and marked with an indelible pencil. The animals were anesthetized with ether, placed in the glass tube, and the thyroid area positioned carefully over the counter. A minimum of 1200 counts was obtained at each determination. To assess the accuracy of this method, multiple counts were performed on six animals and the coefficient of variation found to be $6.6 \pm 2.2\%$.

To correct for extrathyroidal radioactivity in the neck, the neck and chest circumferences were measured in 6 young and 6 old animals, and from the ratio of these measurements, extrathyroidal I^{131} was calculated as follows: the counting rate depends on the amount of tissue "seen" by the counter; this will be a cylinder of length equal to the diameter of the neck or chest; the counting rates for each may be calculated by integrating the counts obtained over the two cylinders, assuming that the counting rate varies inversely with the square of distance. Measurements on a phantom have shown this assumption to be valid.

Let

N 1 = counts over neck (extrathyroidal)
N 2 = counts over chest
T 1 = diameter of neck
T 2 = diameter of chest
r = distance of a disc of tissue from skin surface
R = distance of counter from skin surface
n = number of counts per minute per ml. of tissue at 1 cm.
A = area of counter aperture.

Then

$$N_1 = nA \int_0^{T_1} \frac{dr}{(r + R)^2}$$

and

$$N_2 = nA \int_0^{T_2} \frac{dr}{(r + R)^2}.$$

Integrating and taking the ratio

$$\frac{N_1}{N_2} = \frac{T_1(R + T_2)}{T_1(R + T_1)}.$$

Putting in the experimental values

$$T_1 = 1.9 \text{ cm. } T_2 = 3.8 \text{ cm. and } R = 3.8 \text{ cm.}$$

$$\frac{N_1}{N_2} = 0.65.$$

To test the validity of the foregoing calculation, 6 young and 6 old animals were thyroidectomized, and given tracer doses of I^{131}. The neck/chest ratio of radioactivity at 40 hours was found to be 0.66 ± 0.104 (mean $\pm$ standard error). This figure agrees with that calculated; consequently a chest count was always performed and 0.65 of the chest count subtracted from the counts of radioactivity obtained over the neck.

In all *in vivo* experiments 15 μc. of carrier-free I^{131} were administered intraperitoneally. The initial count was made 40 hours after injection, since over 95% of the thyroidal radioiodine is organically bound at this time (8). Counts were made at 24 hour intervals thereafter, and expressed as a percentage of the initial count. All counts of radioactivity were corrected for physical decay to the time of the initial count.

In Vitro Studies: The 24 hour uptake of I^{131} was determined by estimating the radio-

TABLE 1. THYROID GLAND WEIGHTS IN YOUNG AND SENESCENT RATS

Age	Number of animals	Body weight		Thyroid weight		
		Actual	Body body weight$^{3/4}$	Actual	Per 100 gm. body weight	Per (100 gm. body weight)$^{3/4}$
		(gm.)	(gm.)	(mg.)	(mg.)	(mg.)
4– 5 months	37	186 ± 2.5	50.37 ± 0.50	18.27 ± 0.22	9.93 ± 0.25	11.54 ± 0.275
24–25 months	38	267 ± 3.1	66.05 ± 0.55	23.43 ± 0.49	8.70 ± 0.21	11.26 ± 0.282
P value				P < 0.001	P < 0.001	0.5 > P > 0.4

Values are Mean ± Standard Error.

activity in the thyroid glands removed from the animals. A tracer dose of carrier free I^{131} was administered and 24 hours later the animal was anesthetized with ether and exsanguinated from the abdominal aorta. The thyroid gland was removed, dissected free of extraneous tissue, weighed on a Roller Smith torsion balance to the nearest 0.2 mg. and digested in 10 ml. of 2N NaOH at 45° C. Aliquots were plated on aluminum planchettes, dried under an infrared lamp, and counted under a 3.5 mg. end-window Beta counter. A 5 μc. standard of I^{131} was treated similarly to correct for physical decay and self absorption.

Iodine determinations were performed on thyroid glands and plasma by a modification of the method of Zak *et al.* (9).

Thyroxine solutions were prepared at pH 9.5 as described previously (4).

The following methods of statistical analysis were used: *t* test, analysis of variance and linear regression (1). A probability of less than 0.05 was considered significant.

RESULTS

Thyroid Weights: (Table 1). The thyroid weight of young and old animals maintained on the iodine deficient diet for 10 days were significantly different, as was to be expected, since the body weights of the old rats were considerably greater than those of the young; the mean thyroid weight was 18.27 mg. for the young and 23.43 mg. for the old. Thyroid weight per 100 gm. body weight was less in the old animals, but thyroid weight per unit of metabolic body size (11) shows no significant difference for age; the mean value for the latter was 11.54 mg. for the young and 11.25 mg. for the old. Thus, per unit of metabolic mass, there is an equivalent amount of thyroid tissue present in young and old animals.

I^{131} Uptakes: The 40 hour uptake of I^{131} measured *in vivo* is presented in Table 2. Fifteen μc. of I^{131} were given to both groups of animals. The actual

TABLE 2. 40 HOUR UPTAKE OF I^{131} IN VIVO FOLLOWING ADMINISTRATION OF 15 μC. OF I^{131}

Age	Number of Animals	40 Hour uptake of I^{131}	
		Actual	Per (100 gm. body weight)$^{3/4}$
		(Counts/minute)	(Counts/minute)
4– 5 months	18	2130 ± 103	1385 ± 68
24–25 months	18	1558 ± 95	801 ± 53
P value		P < 0.001	P < 0.001

Values are Mean ± Standard Error.

TABLE 3. 24 HOUR UPTAKE OF I^{131} MEASURED IN VITRO FOLLOWING ADMINISTRATION OF 5 μC. OF I^{131} PER (100 GM. BODY WEIGHT)$^{3/4}$

Age	Number of animals	24 hour uptake of I^{131}		
		Per total thyroid	Per 10 mg. thyroid	Per (100 gm. body weight)$^{3/4}$
		(Per cent)	(Per cent)	(Per cent)
4– 5 months	11	22.5±1.2	12.2±1.0	14.7±0.9
24–25 months	12	18.8±0.8	9.1±0.5	9.3±0.4
P value		0.02>P>0.01	0.02>P>0.01	P<0.001

Values are Mean ±Standard Error.

uptake and the uptake per unit of metabolic mass were significantly greater in the young animals.

The 24 hour uptakes of I^{131} are presented in Table 3. In these experiments the I^{131} was administered on the basis of the metabolic mass; the dose given was 5 μc. per (100 gm. body weight)$^{3/4}$. Again the 24 hour uptake was significantly greater in the young animals. The values for the young were 22.5±1.2%, and for the old 18.8±0.8%. When uptake was related to thyroid weight and to metabolic mass the differences between the two groups were even greater.

Biologic Decay of Thyroid Radioactive Iodine: The release of organically bound radioiodine from the thyroid follows an exponential curve. When the data of the present experiments (Table 4) are plotted on semi-logarithmic paper straight lines result (Fig. 1). It is apparent that the rate of release of radioiodine was more rapid in the young. Analysis of the variance indicates that the differences observed were significant ($P < 0.001$). The biologic half life for the young was 3.1 days and for the old 4.0 days.

It is possible to calculate from each decay curve the proportional daily diminution rate of thyroid radioiodine. The formula of Albert was used (12):

$$\frac{\text{Log } Ct_1 - \text{log } Ct_2}{0.434(t_2 - t_1)}.$$

Ct_1 represents counts of thyroid radioactivity observed at 40 hours and Ct_2 counts observed on day 4. The values obtained in 11 old and 9 young

TABLE 4. BIOLOGIC DECAY OF THYROID RADIOACTIVE IODINE IN YOUNG AND SENESCENT RATS

Age	Number of animals	Radioactivity remaining in thyroid (Per cent of initial count)			
		Day 1	Day 2	Day 3	Day 4
4– 5 months	9	79.0±2.06	65.0±2.91	52.3±2.88	41.1±2.12
24–25 months	11	82.2±2.30	70.2±2.47	60.1±2.72	49.9±2.44

Analysis of Variance $P < 0.001$.
Values are Mean ±Standard Error.

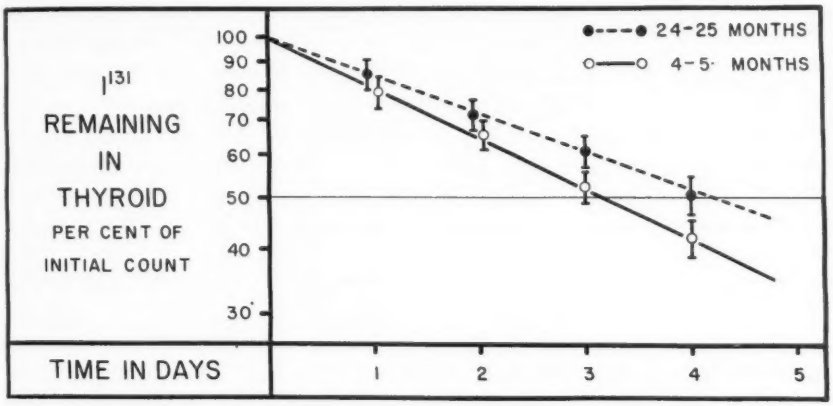

FIG. 1. Biologic Decay of Thyroid Radioactive Iodine in young and senescent rats. The biologic half life for the young was 3.1 days and for the old 4.0 days.

animals were $17.7 \pm 1.25\%$ for the old and $22.5 \pm 1.34\%$ for the young This difference was statistically significant.

Thyroid Secretion Rate: It is generally accepted that exogenously administered thyroxine suppresses the output of thyrotrophin by the pituitary and, as a result, thyroid hormone secretion is reduced. It has been shown (13) that a large dose of thyroxine causes almost complete suppression of release of thyroid I^{131}. This principle has been used to estimate thyroid secretion rate (14) and was applied here for this purpose. Initial readings of thyroid radioiodine were obtained in four paired groups of young and old animals, and doses of 0.75, 1.25, 1.75 or 2.25 μg. of *l*-thyroxine per (100 gm. body weight)$^{3/4}$ were administered subcutaneously, daily, to each pair respectively. Thyroid radioactivity was measured daily for 4 subsequent days (Table 5) and the day 4 value plotted against the dose of thyroxine admin-

TABLE 5. THYROID SECRETION RATE IN YOUNG AND SENESCENT RATS MEASURED BY DOSE OF THYROXINE REQUIRED TO SUPPRESS MAXIMALLY, THE DISCHARGE OF THYROID RADIOACTIVE IODINE

Daily dose of *l*-thyroxine per (100 gm. body weight)$^{3/4}$	Radioactivity remaining in thyroid on day 4 (Per cent of initial count)			
	Animals of 4–5 months		Animals of 24–25 months	
	Number	Per cent	Number	Per cent
(μg.)				
0.75	6	58.0 ± 2.62	6	72.8 ± 3.22
1.25	6	64.2 ± 4.20	5	78.8 ± 3.36
1.75	10	77.6 ± 1.25	8	89.4 ± 1.77
2.25	5	90.0 ± 3.10	5	88.9 ± 1.50
5.00	5	93.4 ± 3.10	5	91.0 ± 2.00

Analysis of Variance for Age $P < 0.001$.
Values are Mean $\pm$ Standard Error.

istered. The response to 5 μg. of *l*-thyroxine per (100 gm. body weight)$^{3/4}$, a dose far in excess of the secretion rates determined by the goitrogen assay, was considered maximal and the smallest dose capable of inducing a similar response was taken to represent the thyroid secretion rate.

Figure 2 indicates that straight line relationships exist for young and senescent animals at the dose levels of 0.75, 1.25 and 1.75 μg. The lines of best fit to the observed points were calculated by the method of least squares. In the senescent animals 1.75 μg. caused maximal suppression of

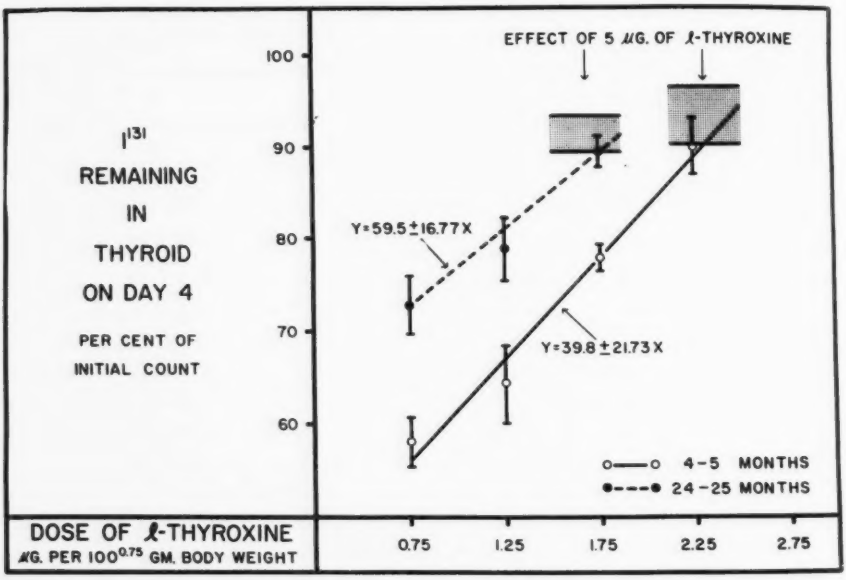

FIG. 2. Thyroid secretion rate in young and senescent rats measured by dose per (100 gm. body weight)$^{3/4}$ of *l*-Thyroxine required to suppress maximally, the discharge of Thyroid Radioactive Iodine. The response to 5 μg. of *l*-Thyroxine was considered maximal. In the senescent animals maximal suppression was obtained with 1.75 μg. of *l*-Thyroxine and in the young with 2.25 μg.

I^{131} release, the response being the same as that obtained with 2.25 μg. and 5 μg. In the young animals maximal suppression was not obtained until the 2.25 μg. dose level. Thus the secretion rate for young animals was estimated to be 2.25 μg. of *l*-thyroxine per (100 gm. body weight)$^{3/4}$ and for the senescent 1.75 μg. Analysis of the variance at the dose levels of 0.75, 1.25 and 1.75 μg. of thyroxine indicates a significant difference for age. There was no significant deviation of the lines from parallelism ($0.7 > p > 0.5$). Therefore the use of this assay as a means of evaluating thyroid secretion rates in the two age groups appears to be valid.

The argument might be raised that even though the thyroxine was given

TABLE 6. THE EFFECT OF THE SAME DOSE OF *L*-THYROXINE (2.5 μG.) ON THE RELEASE OF THYROID RADIOACTIVE IODINE IN YOUNG AND SENESCENT RATS

Age	Number of animals	Body weight	Radioactivity remaining in thyroid on day 4
		(Gm.)	(Per cent of initial count)
4– 5 months	8	198 ± 3.3	72.5 ± 3.1
24–25 months	8	204 ± 5.0	85.2 ± 3.1
P value		0.7 > P > 0.6	0.02 > P > 0.01

Values are Mean ± Standard Error.

on the basis of metabolic mass, the older and heavier animals received more thyroxine and would therefore have more radioactive iodine remaining in the thyroid for this reason alone. In order to evaluate this, healthy animals of 4 and 24 months of the same body weight (198 ± 3.3 gm. and 204 ± 5.0 gm. respectively) were chosen (Table 6). In both groups 2.5 μg. of *l*-thyroxine (equivalent to 1.5 μg. per (100 gm. body weight)$^{3/4}$ which is slightly less than the calculated secretion rate for old animals, were administered daily. The percent activity remaining in the thyroid on day 4 was 72.5 ± 3.1 for the young and 85.2 ± 3.1 for the old; this difference is statistically significant. In young and old animals of the same body weight therefore, the release of radioiodine from the thyroid following the administration of the same dose of thyroxine was less in the senescent rats. This supports the contention that differences that were observed in animals of different weights were true differences in secretion rates and not an artefact due to differences in body size.

Thyroid Iodine: (Table 7). In these studies young and old animals were placed on the iodine deficient diet for 10 days as in the previous experiments. The total iodine content and the iodine content per unit of thyroid tissue were greater in the older animals. The iodine content of 10 mg. of thyroid tissue per (100 gm. body weight)$^{3/4}$ was equal in both groups. The proportion of total thyroid iodine which was protein bound was also the same in both groups.

Plasma Iodine: There was no significant age difference in the concentrations of plasma protein bound or inorganic iodine (Table 8). The protein bound iodine was 3.36 ± 0.18 μg./100 ml. for the young animals and 3.60 ± 0.15 for the senescent.

TABLE 7. IODINE CONTENT OF THYROID GLANDS IN YOUNG AND SENESCENT RATS

Age	Number of animals	Body weight	Thyroid weight	Thyroid iodine content			
				Total	Per 10 mg. thyroid	Per 10 mg. thyroid per (100 gm. body weight)$^{3/4}$	Protein bound fraction
		(gm.)	(mg.)	(μg.)	(μg.)	(μg.)	Per cent of total
4– 5 months	13	190	19.1	11.22 ± 1.11	5.96 ± 0.64	3.70 ± 0.37	92.2
24–25 months	12	267	23.6	20.20 ± 2.34	8.27 ± 0.87	3.95 ± 0.40	90.6
P Value				0.01 > P > 0.001	0.05 > P > 0.02	0.5 > P > 0.4	

Values are Mean ± Standard Error.

TABLE 8. PLASMA IODINE CONTENT OF YOUNG AND SENESCENT RATS

Age	Number of animals	Plasma iodine		
		Protein bound	Inorganic	Total
		(μg./100 ml.)	(μg./100 ml.)	(μg./100 ml.)
4– 5 months	8	3.36 ± 0.18	0.46 ± 0.13	3.82
24–25 months	9	3.60 ± 0.15	0.50 ± 0.20	4.10
P Value		0.4 > P > 0.3	0.9 > P > 0.8	

Values are Mean ± Standard Error.

DISCUSSION

The use of metabolic body size as a reference standard for thyroid function has been discussed in a previous communication (4). Heat production in animals of various body sizes has been found to be more closely related to the $\frac{3}{4}$ power of body weight than to surface area as calculated from the $\frac{2}{3}$ power of body weight. It was thought that this parameter of metabolism would serve as a reasonable basis for comparing thyroid function in animals of different body sizes inasmuch as this gland plays a vital role in the heat production of mammals. In these experiments, thyroid weight per unit of metabolic mass was the same in the two age groups, and this provides further justification for relating thyroid function to metabolic mass rather than to body weight or surface area.

The 40 hour uptake of I^{131} by the senescent thyroid was less than that by the young (Table 2). The rate of renal excretion of I^{131} has been found to be diminished during ageing (15), and this might lead to retention of stable iodide, increasing the size of the iodide pool in the senescent animals. This factor by itself could account for the diminished uptake of I^{131} by the senescent thyroid. More I^{131} was therefore administered to the old animals by calculating the dose on the basis of metabolic body size. The 24 hour uptake was still significantly diminished in the senescent rats. The fact that I^{131} uptake per 10 mg. of thyroid tissue was diminished in the senile animal suggests that unit for unit the old thyroid is less capable of taking up iodide. Furthermore, per unit of metabolic mass I^{131} uptake by the senile thyroid was less, whereas the thyroid weight was equal.

The biologic decay of thyroid radioiodine is not an absolute measure of thyroid secretion rate but serves as an index of secretion rate. The biologic half life was 4 days for the senescent animals and 3.1 days for the young animals (Fig. 1). However, since the senescent thyroid has a greater total iodine content, the longer half life could be attributed to this rather than to diminished secretion rate.

In the secretion experiments the specific activity of thyroid radioiodine was probably of no consequence at the dose levels of 1.75 μg. and 2.25 μg. in the senescent rat, and at 2.25 μg. in the young. At these dose levels maximal suppression had taken place and there was practically no release

of radioiodine. Thus at the 1.75 μg. dose level there was maximal suppression in the senescent thyroid whereas significant release of radioiodine continued from the young gland (Fig. 2). The daily secretion rate for the senescent animals was estimated to be 1.75 μg. of *l*-thyroxine per (100 gm. body weight)$^{3/4}$ and for the young 2.25 μg. These compare favourably with the values of 1.6 μg. and 2.4 μg. respectively obtained in this laboratory under similar circumstances by the goiter prevention assay (4). Similar values of 2.31 to 2.56 μg. of *l*-thyroxine/100 gm. body weight per day were obtained by Reineke and Singh (16) using the method herein described in adult female rats of unstated age.

The iodine content of 10 mg. of thyroid per (100 gm. body weight)$^{3/4}$ was equal in young and old animals of varying metabolic mass (Table 7). Thyroid secretion rate was measured in young and old animals of equal metabolic body size and thyroid weight on the assumption that the total thyroid iodine content and specific activities of thyroid radioiodine were equal. The data in Table 6 indicate that under these circumstances thyroid secretion rate was less in the senescent animals.

From these observations it is apparent that, in the rat, thyroid secretion rate is diminished during senescence. Since the plasma protein bound iodine does not differ in concentration in the two age groups, one must conclude that the rate of removal of hormonal iodine from its pool is diminished in the aged rat. If the requirement of the target tissues were unchanged during senescence, the old animal would then by hypothyroid. If this speculation were true, there would be a rational basis for thyroid hormone therapy for the aged. If the requirement of the target tissues were diminished due to increased responsiveness or impaired inactivation or excretion of thyroid hormone, the senile animal would not be hypothyroid. In this event, there would be no rational basis for thyroid therapy.

Experiments are being carried out to test the responsiveness of the ageing tissues to thyroid hormone. Preliminary results indicate a greater rise in oxygen consumption in senile than in young thyroidectomized animals following injections of the same dose of thyroxine given in relation to metabolic mass. This is evidence against the existence of a hypothyroid state in senescence and suggests that diminished thyroid secretion may be a homeostatic adaptation to the increased responsiveness of the senescent target tissues, or to diminished inactivation or excretion of thyroid hormone.

SUMMARY

The functional status of the thyroid gland in senescence has been studied. Thyroid weights were greater in rats of 24 to 25 months of age than in those of 4 to 5 months. However, per (100 gm. body weight)$^{3/4}$ they were equal in both groups. The twenty-four and forty hour uptakes of I^{131} and the biologic decay of thyroid radioactive iodine were significantly diminished

in the senescent animals; the biologic half life for the old animals was 4.0 days and for the young 3.1 days.

The daily thyroid secretion rate for old animals was estimated to be 1.75 μg. of *l*-thyroxine per (100 gm. body weight)$^{3/4}$ and for young animals 2.25 μg.

The concentration of circulating PBI was not significantly different in young and old animals.

Preliminary experiments are cited as evidence against the occurrence of hypothyroidism as a natural consequence of senescence. They suggest that the diminished thyroid secretion rate is a homeostatic adaptation to the increased responsiveness of the target tissues, or to impaired inactivation or excretion of thyroid hormone in the senescent rat.

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A PRECISE METHOD FOR THE BIOASSAY OF URINARY GONADOTROPIN WITH A SUGGESTION FOR A STABLE, EASILY AVAILABLE REFERENCE STANDARD[1]

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THE bioassay of urinary gonadotropin (UG) has presented some difficulties for many years. For reviews of the literature the reader is referred to Catchpole (1), Albert (2) and Loraine (3). The absence of a stable primary standard together with the use of relatively small numbers of animals without precise statistical evaluation has frequently led to rough estimates of the titer of this material in human urine, rather than rigidly defined meaningful quantitative determinations. This work was oriented specifically to improving both the precision of the method as well as establishing a reference material which would be easily available, stable, and reproducible. The use of a standard preparation derived from a pool of normal women's urine was suggested by Catchpole (1). More recently Albert (4) suggested that an absolute unit of measurement could be obtained by employing a reference material derived from the urine of healthy normal menstruating women, from the urine of postmenopausal women, or from urine of men. Since the end-point of uterine hypertrophy in the intact immature mouse appears to be satisfactory for estrogen bioassay (5) and since the response to UG is actually due to stimulation of the uterus caused by the ovarian release of estrogens, the use of estrone as the primary standard is indicated. If this were possible, data from any laboratory could be referred to the crystalline material or, in other words, titers of UG expressed as μg. of estrone equivalent and the values derived from laboratory to laboratory should be strictly comparable. From a theoretical bioassay point of view this procedure is possible if it can be shown that the log dose response curves described by UG and estrone are parallel, reproducible, and the index of precision favorable. Further, the possibility exists that different strains of mice may vary as to the comparative sensitivity of the uterus to estrone and the ovary to UG. If in all strains of mice the comparative sensitivities of uterus and ovary to estrogens and UG, respectively, re-

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mained constant, then the activity of UG could be expressed in terms of estrone. If the relative sensitivities of these end-points does not remain constant from strain to strain then the method would only yield comparative results. Our studies have indicated that when mice of three different strains are used similar results are obtained and that the method has an acceptable precision and sensitivity.

METHODS AND MATERIALS

Conditions for Bioassay

20–22-Day-old female mice (weighing 6–8 gm.) of the Swiss albino strain obtained from Taconic and Rockland Farms and Armer Enterprises were employed in these studies. The mice were injected subcutaneously and the total dose of estrone was contained in 0.3 ml. of corn oil; 0.1 ml. being administered once daily for three days. The total dose of UG (prepared by method of Heller and Chandler, 6) was dissolved in 0.5 ml. of distilled water; 0.25 ml. was injected subcutaneously once on day 1 and again on day 2. The mice did not receive UG on day 3. Twenty-four hours after the last estrone injection (48 hours after the last UG injection) the animals were autopsied. The uteri were separated from the vagina by cutting through the cervix, the surrounding tissue was stripped off and the utero-tubal junctions severed. The uteri were then weighed fresh on a Roller-Smith torsion balance after pressing out the intrauterine fluid on blotting paper. At autopsy body weights were determined to the nearest gram and the results expressed as the ratio of milligrams of uterus per gram of body weight. The experimental design was such that a solvent control and three concentrations of the unknown (UG) and three concentrations of the standard (estrone) were run simultaneously. The slopes and the test for parallelism of the respective log dose responses were calculated by the method of Fisher (7). Other statistical methods employed in this study have been described by Irwin (8), Bülbring (9), and Emmens (10).

RESULTS AND CONCLUSIONS

Table 1 summarizes our experience with nine sample bioassays. The first three assays compare the response to UG obtained from normal men's urine to that of estrone. Assay 4 is concerned with a sample of UG derived from postmenopausal urine. Assays 5, 6 and 7 are on a single pooled sample of postmenopausal urine employing three different strains of mice. Assays 8 and 9 represent the response from a 6 day pool of postmenopausal urine assayed on two different strains of mice. It was observed that in seven out of nine trials the respective slopes of the UG and estrone were not significantly different. The index of precision was extremely favorable and consistent, varying from 0.12–0.14. The three samples of men's urine gave values of 1.04, 1.3 and 1.7 μg. of estrone equivalent per day. Postmenopausal urine (assays 4, 5, 6 and 7) gave values ranging from 2.6 to 27 μg. estrone equivalent per day. Of particular significance is the fact that when a preparation was assayed on three occasions (assays 5, 6 and 7) using three different strains of mice, the UG titer was found to be 23, 21 and 27 μg. of estrone equivalent per day, respectively. The close agreement of these values together with the small error range points to the fact that

TABLE 1. THE BIOASSAY OF URINARY GONADOTROPIN

Assay No.	Source of Swiss albino mice	Estrone standard		Urinary gonadotropin		Combined slopes b	Index of precision λ	Significance of difference of slopes t (P)	Concentration of urinary gonadotropin estrone equivalent, μg./day (Range at P=0.95)
		Total dose μg.	No. of mice	Total dose, hours equivalent (source)	No. of mice				
1	Taconic Farms	0.05; 0.1; 0.2	42	0.75; 1.5; 3 (men)	41	4.0	0.12	0.2 (>0.5)	1.7 (1.5–1.9)
2	Taconic Farms	0.05; 0.1; 0.2	40	1.5; 3 (men)	19	6.3	0.12	0.5 (>0.5)	1.3 (1.16–1.47)
3	Armer Enterprises	0.1; 0.2; 0.4	42	1; 2; 4 (men)	33	2.9	0.13	2.6 (0.01)	1.07 (1.23–0.93)
4	Rockland Farms	0.05; 0.1; 0.2	42	1/16; 1/8; 1/4 (post-menopausal from one patient	30	3.7	0.11	2.1 (0.65)	13.13 (14.8–11.6)
5	Rockland Farms	0.05; 0.1; 0.2	49	1/20; 1/10; 1/5 (post menopausal)	48	4.2	0.14	1.28 (>0.1<0.5)	23 (20–26)
6	Taconic Farms	0.05; 0.1; 0.2	51	1/20; 1/10; 1/5 (post-menopausal)	50	4.7	0.13	0.2 (>0.5)	21 (18–23)
7	Taconic Farms	0.05; 0.1; 0.2	51	1/20; 1/10; 1/5 (post-menopausal)	51	3.5	0.14	0.5 (>0.5)	27 (23.7–30.8)
8	Rockland Farms	0.1; 0.2; 0.4	41	1/2; 1; 2 (pooled post-menopausal urine)	24	3.7	0.13	0.9 (>0.5)	3.3 (3.8–2.8)
9	Armer Enterprises	0.1; 0.2; 0.4	40	1/2; 1; 2 (pooled post-menopausal urine)	22	3.4	0.14	0.8 (>0.5)	2.6 (3.1–2.2)

this assay is indeed quite precise. In this study we have employed a total of between 69 and 102 mice per assay. For routine studies it is possible that the use of a total of 36 mice (18 on the standard and 18 on the unknown) will result in the determination of the relative potency with an accuracy of approximately ±25% at P=0.95.

DISCUSSION

On the basis of the data presented in this paper we suggest that for precise evaluation of UG a method such as the one described here is highly suitable. Estrone can serve as a convenient, reproducible, easily-available standard and has been shown to give a dose-response curve, under the conditions described, which is parallel to that obtained with UG preparation. This method provides a sensitive and highly accurate bioassay for UG. Differences in UG content of urine of the order of 35% could be easily established.

Our studies indicate that in at least three strains of mice the relative responsiveness of the uterus to estrogen and the ovary to UG can be considered to be similar. If this relationship remains unchanged as more strains are studied the use of the method could lead the way to a reproducible means for the determination of UG, consequently, data from

laboratory to laboratory could be easily compared. If different strains give different relative response ratios for the uterus and ovary, the method will provide means by which UG could be determined in a single laboratory with a high degree of confidence but absolute values from laboratory to laboratory could not be compared. As a consequence, comparative values from different laboratories could be highly meaningful.

The method as described here for the determination of UG, as is true of many previously published methods employing the weight response of the rodent uterus, only measures the ability of these crude urinary concentrates to stimulate estrogen production by the ovary and does not add any precise information on the question of the FSH and LH content of urine.

SUMMARY

A method has been described for the precise evaluation of urinary gonadotropin (UG) which employs the mouse uterine response and consists in comparing the log dose-response of the unknown (UG) to the log dose-response of the standard (estrone) run simultaneously. The use of a total of 80 mice permits the evaluation of the relative potency with an accuracy of ± 13 per cent at $P = 0.95$. If an error range of ± 25 per cent ($P = 0.95$) is acceptable a total of 36 mice would give the desired answer. The accuracy of the method is illustrated by the favorable index of precision, λ, which varied in nine trials between 0.12 and 0.14. Estrone can be used as a primary standard for the determination of UG on the basis that conditions have been found, and are herein described where the log dose response curves of estrone and UG are parallel and on the basis that estrone is an easily available, highly stable reference material.

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BLOCKADE OF THE RELEASE OF PITUITARY OVULATING HORMONE IN THE RAT BY CHLORPROMAZINE AND RESERPINE: POSSIBLE MECHANISMS OF ACTION[1]

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SPONTANEOUS ovulation in the female rat is known to involve a neurogenic timing factor which activates the adenohypophysis to release ovulating hormone (LH or total gonadotrophin). Under controlled lighting conditions, the intrinsic stimulus responsible for such pituitary activation occurs between the hours of 2 P.M. and 4 P.M. on the day of proestrus, and it may be interrupted by various neural blocking agents (1–3).

Recently, electroencephalographic (EEG) studies have been conducted in the rat to determine the mechanisms by which such known ovulation-blocking agents as atropine, nembutal and morphine inhibit the release of ovulating hormone (4). It was observed that dosages of these compounds which block ovulation have several common effects on the electrical activity of the brain. All induce high-amplitude, slow-wave electrical activity in cortical and subcortical areas, and all raise the threshold of EEG arousal on direct stimulation of the midbrain reticular formation. From these observations, two alternative hypotheses have been proposed to relate the reticular activating system and hypothalamic excitation of the adenohypophysis: (a) the role played by the reticular formation may be "permissive" in the sense that it maintains the hypothalamus in an active state while not being directly concerned with specific excitation; or (b) the role may be a specifically excitatory one in which the pituitary-activating stimulus is passed through interneuronal pathways to the reticular activating system and is then relayed to the hypothalamus. Regardless of which hypothesis may be valid, it would appear essential that an "active" reticular activating system be present for stimulation of the hypothalamic centers controlling adenohypophyseal function.

Since these earlier observations, two compounds have been developed with the interesting property of producing sedation without hypnosis or anesthesia: reserpine and chlorpromazine. Several reports have indicated that reserpine and chlorpromazine interrupt estrus cycles and alter ovarian

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structure in the rat (5–7). These effects have been loosely attributed to inhibition of the release of pituitary gonadotrophin. However, no specific studies have been conducted with these agents on the neurally-controlled ovulatory mechanism in the rat, nor on how pituitary inhibition may be related to changes in the electrical activity of the central nervous system. The present investigation seeks answers to these questions.

We have studied the effects of chlorpromazine and reserpine on the release of pituitary ovulating hormone and on the electrical activity of the central nervous system and the EEG arousal mechanism following stimulation of the midbrain reticular formation. These studies, therefore, permit one to evaluate not only the action of reserpine and chlorpromazine in the hypothalamic control of the release of ovulating hormone, but also the role of the reticular activating system in the ovulatory mechanism of the rat. The latter aspect is of particular interest since chlorpromazine has been reported to depress reticular activity and slow the EEG record (8–11), while reserpine fails to alter reticular activity and may actually induce central arousal (9, 12).

The results show that reserpine and chlorpromazine block ovulation if injected prior to 2 P.M. on the day of proestrus, but fail to exert such blockade if injected after 4 P.M. Doses of chlorpromazine, which are effective in blocking ovulation, induce high-amplitude slow waves in the cerebral cortex and subcortical areas of the brain and depress midbrain reticular formation activity, as evidenced by an increased threshold of EEG arousal. Reserpine, on the other hand, fails to alter midbrain reticular activity but appears to inhibit afferent input into the reticular activating system from the medullary brain stem and cerebellum. These results have been reported previously in abstract form (13, 14).

MATERIALS AND METHODS

Sexually mature Sprague-Dawley female albino rats were maintained in a temperature-controlled, artificially illuminated room with 14 hours of light and 10 hours of dark each day. Noon, colony time, occurred midway in the light period, and the critical timing period, between 2 and 4 hours later. Rats were purchased at a weight of 225 gm. and kept under these lighting conditions about 30 days before use. Prior to experimentation daily vaginal smears were taken for a 3-week period to insure cyclic regularity. Under these conditions the rats used in this investigation were found normally to run 5-day cycles.

Chlorpromazine (Thorazine)[2] was dissolved in Ringer's solution prior to administration, whereas reserpine (Serpasil)[2] was brought to correct concentration with special Serpasil injectable solution. Four groups of rats were used in the chlorpromazine study. Since the critical timing period falls on proestrus between the hours of 2 P.M. and 4 P.M. (15), one group of animals was injected intravenously with 5 mg./kg. of chlorpromazine between 1 P.M. and 2 P.M., and a second group at 4 P.M. on that day. A third group of rats received 50 mg./kg. subcutaneously between 1 P.M. and 1:30 P.M. on proestrus, to

[2] Grateful acknowledgment is made to Ciba Pharmaceutical Products, Inc. for the Serpasil and to Smith, Kline and French Laboratories for the Thorazine.

allow sufficient time for absorption. The remaining animals received 5 mg./kg. of chlorpromazine intravenously between 1 P.M. and 2 P.M., but were maintained until the second day after treatment. Similarly, 4 groups of rats were employed in the reserpine study. However, due to the latency in response following injection, 2 groups of animals received either 1.0 mg./kg. or 0.1 mg./kg. of reserpine subcutaneously between 12 and 12:15 P.M. and a third group, the 1.0 mg./kg. dose at 4 P.M. on the day of proestrus. The final group of rats was injected intravenously with 0.2 mg./kg. between 12 and 12:15 P.M. on the day of proestrus.

All animals, with the exception of the one group of chlorpromazine-treated rats, were sacrificed with ether and autopsied the first morning after treatment. At autopsy, the oviducts were excised and the ampullae examined for tubal ova, and the ovaries and uteri were examined as described by Everett (16–18). Surgical preparation, EEG recordings, stimulation techniques and parameters were similar to the methods described by Sawyer *et al.* (4). The dosages and routes of administration used in this phase of the experiment will be described below.

RESULTS

Sedation was the most obvious symptom in rats receiving reserpine or chlorpromazine. A great degree of sedation was observed in the chlorpromazine-treated animals, and it occurred within 15–30 minutes after treatment, depending on the route of administration. In contrast, a period of 60–90 minutes was required for reserpine to produce similar sedation regardless of the route of administration. Both groups of rats could be awakened, but they appeared indifferent to external stimuli such as handling or pinching of the foot or tail. Miosis and ptosis occurred in reserpine-treated rats, but not in animals receiving chlorpromazine. Neither exophthalmos nor catatonia was observed in either group. Signs of recovery appeared 24–48 hours after injection of either drug.

The effects on ovulation are summarized in Table 1. It is apparent that neither compound is effective if injected at the end of the "critical period," 4 P.M., but that either agent is highly active in blocking ovulation if in-

TABLE 1. EFFECTS OF RESERPINE AND CHLORPROMAZINE ON OVULATION IN THE RAT

Drug	Dosage, mg./kg.	Time of injection at proestrus (day 1)	Number of animals	Results at autopsy on on day 2 (estrus)	
				No. ovulated	% blocked
Reserpine	1.0 s.c.	12–12:15 P.M.	10	1	90
		4 P.M.	6	6	0
	1.0 s.c.	12–12:15 P.M.	6	6	0
	0.2 i.v.	12–12:15 P.M.	6	3	50
Chlorpromazine	5.0 i.v.	1–2 P.M.	8	0	100
		4 P.M.	6	6	0
	50.0 s.c.	1–1:30 P.M.	6	0	100
				Results at autopsy on day 3	
	5.0 i.v.	1–2 P.M.	5	0	100

jected at such a time, and in such a dosage as to induce marked sedation by 2 P.M. With reserpine, 0.1 mg./kg. s.c. was ineffective, while 0.2 mg./kg. i.v. blocked ovulation in half the cases. With chlorpromazine, 50 mg./kg. s.c. and 5 mg./kg. i.v. were equally potent. Chlorpromazine was seen to retain its effectiveness more than 24 hours in all 5 animals which were autopsied on day 3.

In view of the evidence that reserpine and chlorpromazine can block ovulation in the rat, investigations were undertaken to determine the manner in which, and the sites at which, these drugs act in the central nervous system to effect such blockade.

1. *Control electroencephalographic activity.* Control EEG activity was recorded in 18 rats by means of bipolar electrodes placed in various subcortical regions, including the brain stem, and by unipolar electrodes in the frontal and parietal cortical areas. The normal resting record obtained from these areas was generally of the low-voltage (25–50 μv), high-frequency variety, although periodically spontaneous 5–7/sec. rhythms were observed in the hippocampus and regions receiving hippocampal projections, such as the parietal cortex or mammillary bodies.

Since the unanesthetized, curarized rat, unlike the rabbit or cat, fails to show high-amplitude slow waves except when maintained for long periods (4–6 hours), we have utilized, as our basis of determining EEG arousal in this already "alert" animal, the induction of 5–7/sec. waves by stimulation of the midbrain reticular formation. Such "theta" arousal has been suggested by Green and Arduini (19) to be hippocampal in origin, and it is easily obtained in the rat by low-voltage stimulation of the midbrain reticular formation. A typical electrically-induced arousal in an alert animal is shown in Figure 1. It is noteworthy that similar rhythms can be elicited by painful stimuli such as pinching the foot or tail, and they correspond to behavioral arousal.

2. *Chlorpromazine.* Following 60 to 90 minute periods of recording control EEG activity and measuring thresholds of arousal, chlorpromazine was administered to 8 rats in a dosage known to block pituitary activation. Figure 1 illustrates the effects of 5 mg./kg. of chlorpromazine, injected intravenously. Within 5 minutes after treatment, an increase in amplitude (100–200 μv) and a decrease in frequency (3–4/sec.) of the electrical waves were observed. These EEG alterations appeared initially in the cortical channels, but rapidly involved subcortical areas as well and lasted throughout a 3-hour observation period. Thresholds of arousal to stimulation of the midbrain reticular formation were increased 70 to 100%, and in one animal almost 500% (Figs. 1, 2). This depression of reticular activity occurred within 10 minutes and reached a maximum between 1 and $1\frac{1}{2}$ hours after treatment. Partial recovery from chlorpromazine, as evidenced by a slight decrease in arousal thresholds, was observed 3–4 hours after treatment.

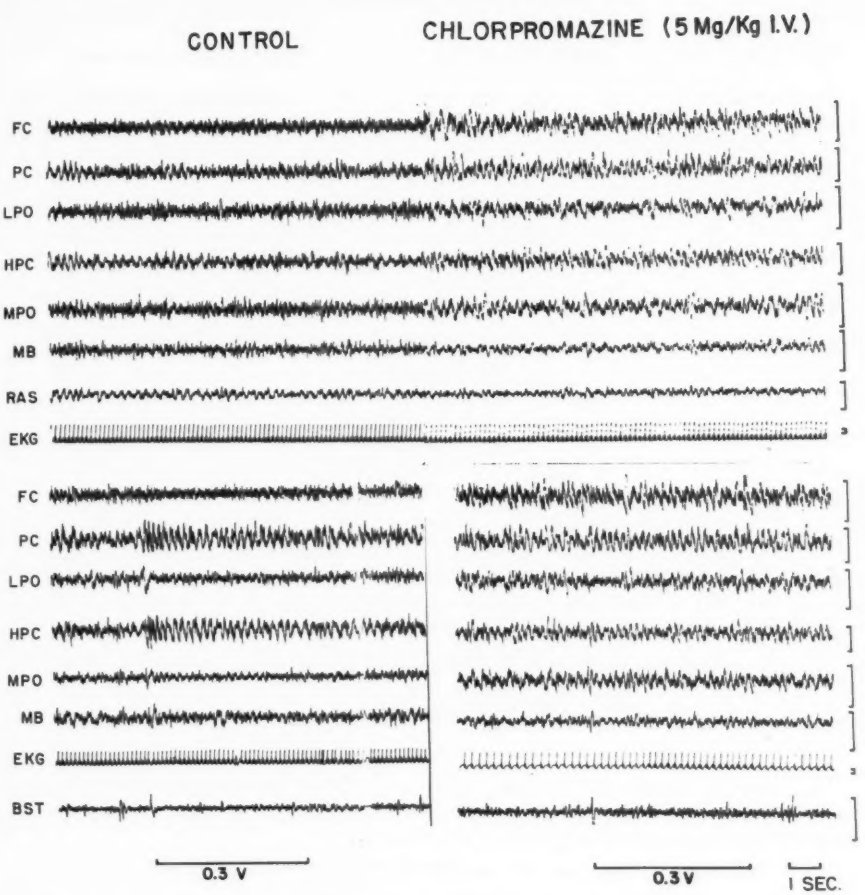

FIG. 1. Changes induced by chlorpromazine (5 mg./kg. i.v.) on EEG activity in the rat and on the threshold of arousal to direct stimulation of the midbrain reticular formation. Stimulation was applied during the underlined periods, (0.3 volt, 0.5 millisecond square waves). Voltage calibration scale in 100 μv. Abbreviations employed in this and subsequent figures: FC, PC, frontal and parietal cortex; LPO, MPO, lateral and medial preoptic areas; D. HPC, V. HPC, dorsal and ventral hippocampus; MB, mammillary body; RAS RF, midbrain reticular formation; Amyg, amygdala; BSt, Br. St., medullary brain stem; Tub, tuberal region; EKG, electrocardiogram; VA, ventral anterior thalamic nucleus.

3. *Reserpine.* Similar recordings were made in 10 rats for 60 to 90-minute control periods (Fig. 3-A), after which a known ovulation blocking dose of reserpine (0.2 mg./kg.) was given intravenously. For the purpose of description, we have categorized the observed EEG effects of reserpine into two phases.

A. *Phase I.* This phase appeared within 50–60 minutes after treatment.

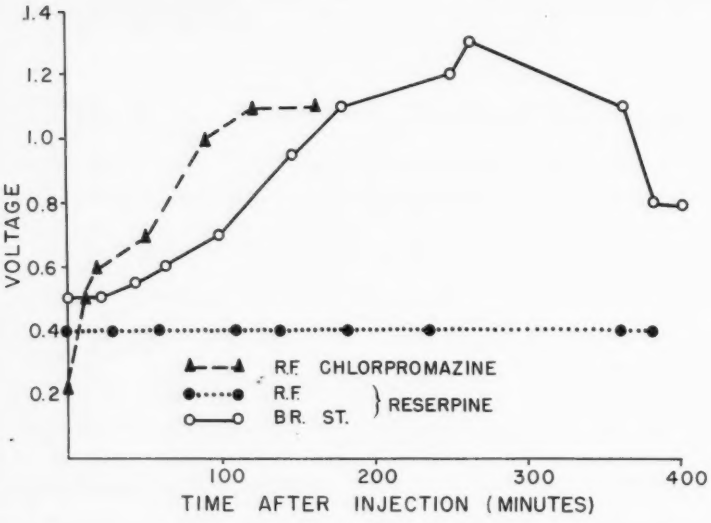

FIG. 2. Changes in thresholds of arousal following injection of chlorpromazine (5 mg./kg. i.v.) or reserpine (0.2 mg./kg. i.v.) and stimulation of either the midbrain reticular formation (RF) or medullary brain stem (Br. St.).

The most obvious change from the normal pattern was the appearance of high-amplitude spikes ranging from 100–200 μv. This is contrasted to the pre-treatment record, in which the maximum amplitude varied between 25 and 50 μv. However, unlike the alteration following chlorpromazine, the frequency remained essentially unchanged (Fig. 3-B). After 10–15 minutes, this pattern was followed by a further change in the EEG which we have designated as Phase II.

B. *Phase II.* This was characterized by marked bursts of 7–8/sec. rhythms which were more noticeable in hippocampal channels, but which also involved the n. ventralis anterior of the thalamus and the parietal cortex (Fig. 3-C). These bursts lasted from 8–15 seconds; they were then replaced for 30–60 seconds by irregular waves before reappearing for another 8–15 seconds. This fluctuation between a fast "theta" rhythm and high-amplitude bursts of less regular activity typified the record throughout the remainder of the experiment (4–6 hours).

In contrast to chlorpromazine, reserpine failed to alter arousal thresholds to direct electrical stimulation of the midbrain reticular formation, even though such thresholds were determined for periods as long as 6 hours (Fig. 2). If, however, arousal was induced by electrical stimulation of areas lateral to the medullary brain stem reticular formation, in the region of the VII nerve and the V nerve nucleus, or by stimulation of the cerebellum in the region of the dentate nucleus, marked changes in arousal thresholds were observed. Whereas 0.5 volt would elicit a "theta" arousal in the parietal cortex prior to treatment, progressively higher voltages were re-

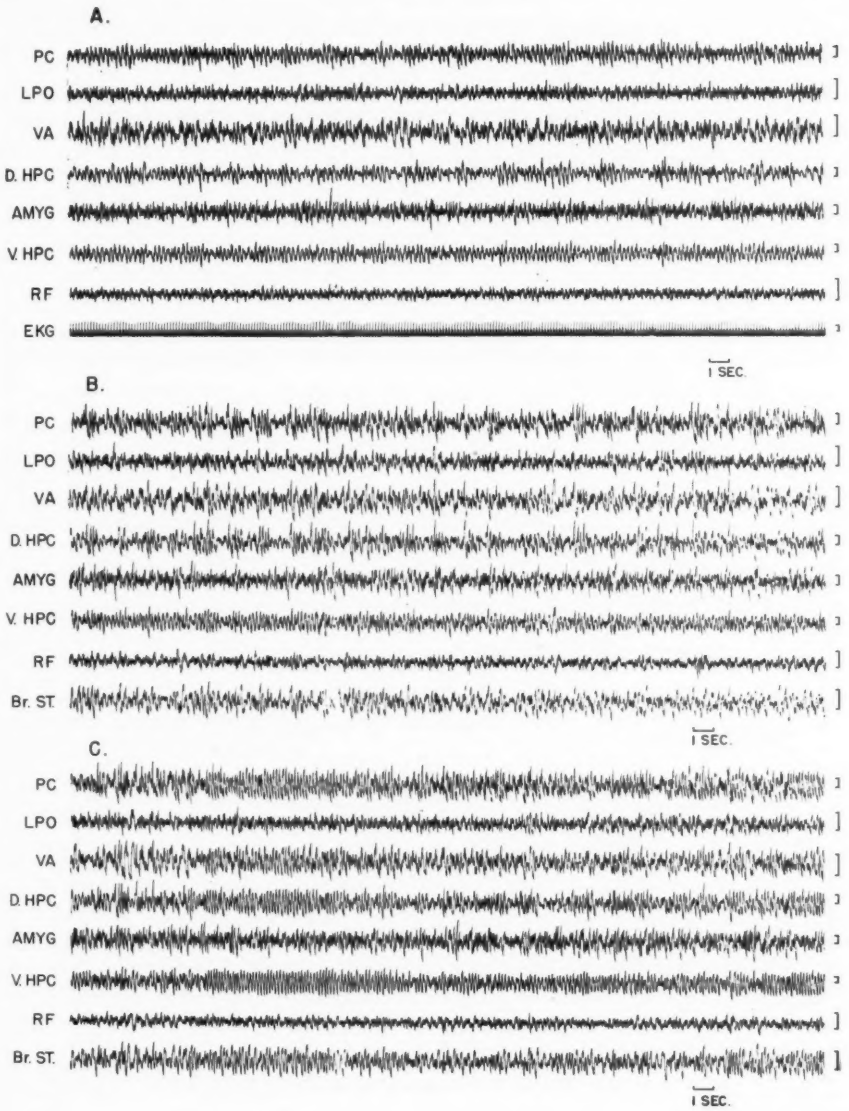

FIG. 3. Effects of reserpine (0.2 mg./kg. i.v.) on the spontaneous electrical activity of the rat cerebral cortex and brain stem. A. Control recording. B. Ten to 15 minutes after reserpine. C. Fifteen to 20 minutes after reserpine.

quired to induce this same arousal following injections of reserpine (Figs. 2, 4). It should be pointed out that in these experiments the midbrain reticular formation was stimulated alternately with either the medullary brain stem or cerebellum, in the same rat. An eventual partial decrease in

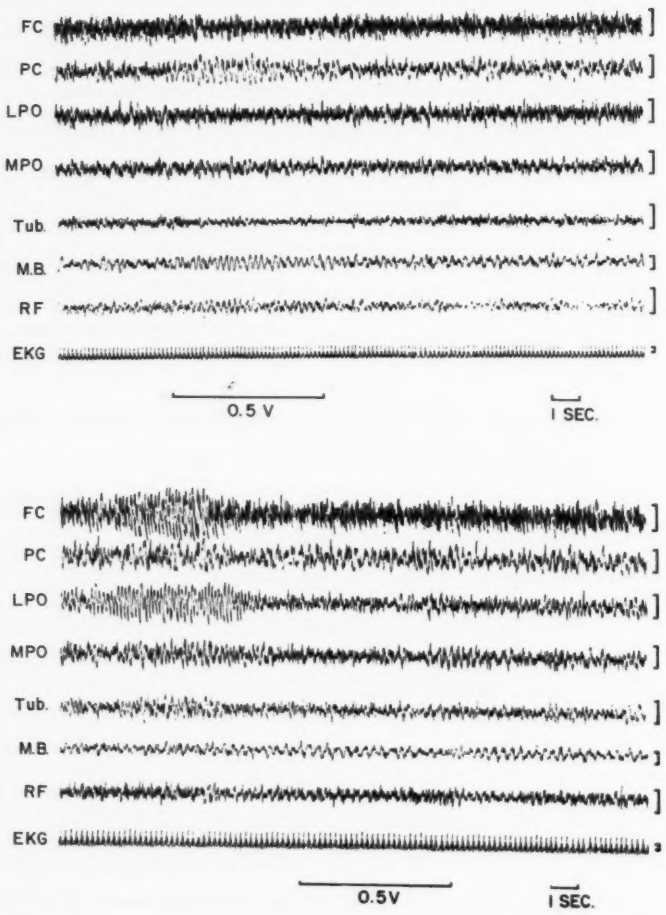

FIG. 4. Effect of reserpine (0.2 mg./kg. i.v.) on the arousal reaction to stimulation of the medullary brain stem. Stimulation during the underlined periods, (0.5 volt, 0.5 millisecond square waves). Upper chart, before reserpine; lower chart, after reserpine.

arousal threshold was observed 4–5 hours after treatment, although a return to original levels was never attained by 6 hours, at which time the experiments were terminated.

DISCUSSION

The ability of reserpine and chlorpromazine to block ovulation in the rat confirms the mounting array of evidence that neurogenic stimulation of the adenohypophysis is necessary for the release of ovulating hormone in a spontaneously ovulating animal. These results further emphasize the importance of the time relationships of the critical period, and the neces-

sity of injecting drugs in sufficient time prior to this period to compensate for latency in response and duration of action of the compound administered. Similar results have been obtained in the monkey (20) and human being (21). The present evidence substantiates the theory that these sexual aberrancies result from an interruption of neurogenic activation of gonadotrophin secretion from the adenohypophysis.

Another phase of adenchypophyseal function, controlled by the hypothalamus and reported to be inhibited at least partially by chlorpromazine and reserpine, is the stress-induced release of ACTH. However, results are controversial with regard to this hormone (22–28). Seemingly, reserpine (29) and chlorpromazine (27) effectively block the stress-induced release of ACTH only when administered under conditions in which their initial stimulatory action is suppressed, as under pentobarbital anesthesia in acute experiments. It is, perhaps, significant that gonadotrophin release is blocked without the need of a possible synergistic action with pentobarbital.

From the electrical recording and stimulation studies, it is apparent that there are marked differences in the effects of chlorpromazine and reserpine on the central nervous system. Chlorpromazine, in doses which effectively block ovulation, induces high amplitude slow waves in cortical and subcortical areas. While these wave forms are not identical to the type observed with the barbiturates, they nevertheless appear to be associated with a depression of the midbrain reticular activating system. Similar depression has been reported for the rabbit (8, 10, 11) and cat (30). Chlorpromazine may, therefore, be categorized with drugs such as atropine, morphine and the barbiturates, which combine the process of inhibiting midbrain reticular formation activity with blockade of the release of pituitary ovulating hormone. The importance of the midbrain tegmentum to cyclic ovulation in the rat has recently received direct confirmation from the work of Critchlow (31). This investigator finds that localized midbrain lesions block ovulation, while the hypothalamus is still responsive to direct electrical stimulation (personal communication).

Reserpine, on the other hand, fails to depress midbrain reticular formation activity yet it is equally effective in blocking ovulation. Bein (32) has shown that an intact connection between medulla and midbrain is essential for reserpine to diminish the pressor carotid-sinus response. Schneider *et al.* (33) have suggested that the predominant parasympathetic syndrome seen with reserpine may be associated with an inhibition of afferent transmission to centrally located sympathetic centers. In our studies, reserpine appeared to inhibit afferent transmission to the medullary reticular formation when stimuli were applied to either the cerebellum or medullary brain stem. Under similar conditions of reserpine blockade in the rabbit, ovulation may still be induced by direct electrical stimulation of the hypothalamus (34). The inference is that the hypothalamus and midbrain reticular

system are "alert" but are inactive due to failure of the afferent input to the reticular formation.

There are other sites and mechanisms of action of the two drugs which merit consideration. Strong doses of chlorpromazine have induced localized amygdaloid seizures in the cat (35). Chlorpromazine is a reasonably effective adrenergic blocking agent (30), and the duration of its pituitary blocking action resembles that of dibenamine (1). Weak but effectively sedative doses of the drug in the cat have little effect on the midbrain reticular system (12). Reserpine has altered certain rhinencephalic thresholds in the rabbit (36), and it has been reported to induce spontaneous rhinencephalic seizures in the cat, as well as enhancing seizure activity in response to amygdaloid stimulation (12). These changes assume significance in view of the demonstrations that amygdaloid stimulation causes ovulation in the rabbit (37) and cat (38) and that surgical lesions in rhinencephalic pathways have interrupted artificially induced ovulation in the rabbit (39).

While multiple sites of action of reserpine and chlorpromazine must be considered as possibly influencing hypothalamic activation of the adenohypophysis, the present experiments suggest that blockade of afferent input to the reticular activating system, or depression of the reticular system itself, may inhibit ovulation. The experiments suggest also that, rather than exerting a "permissive" action on the hypothalamus, the reticular system actually transmits afferent impulses from the lemniscal system to the hypothalamus. This is indicated by the observation that reserpine blocks hypophyseal function by interrupting the afferent input to an active midbrain-hypothalamus complex. The contribution of the rhinencephalon to the activation process has yet to be elucidated.

SUMMARY

The effects of chlorpromazine and reserpine on ovulation in mature cyclic Sprague-Dawley female rats have been studied. In these experiments we have utilized previous observations that neurogenic activation of the pituitary occurs between 2–4 P.M. on the day of proestrus. When either compound was injected prior to 2 P.M. on this day, ovulation failed to occur that night, whereas animals treated after 4 P.M. did ovulate.

Parallel studies were conducted on the manner in which reserpine and chlorpromazine affect the electrical activity of the rat central nervous system and thresholds of arousal, following stimulation of the midbrain reticular formation. Doses of chlorpromazine which were effective in blocking ovulation induced high amplitude slow waves, and depressed midbrain reticular activity as evidenced by increases in arousal thresholds.

In contrast, the EEG record subsequent to reserpine treatment was characterized by a fluctuation between a fast "theta" rhythm that was particularly prevalent in hippocampal channels, and high-amplitude bursts of less regular activity. This drug, however, failed to alter arousal thresh-

olds on stimulation of the midbrain reticular formation, but appeared to inhibit afferent transmission into the reticular activating system from the medullary brain stem and cerebellum.

The manner in which these electrical changes are related to a blockade of ovulation in the rat is discussed.

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BIOASSAY OF THYROTROPIC HORMONE BY WEIGHT RESPONSE OF BOVINE THYROID SLICES[1]

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THE bioassay of thyrotropic hormone (TSH) continues to be an area of active investigation because of the technical difficulty, variability, and limited sensitivity of existing methods. The present one is reported in detail because it is unusually sensitive, economical, and simple, permitting two technicans to obtain 130 or more determinations daily. It shares the limited precision of other methods for assay of TSH.

This assay is based upon previously reported observations (1) of the effect of TSH on the osmoregulation of surviving beef thyroid slices. The final weight of the slices reflects a specific and proportional response to TSH added to the incubation medium.

METHOD

A. *Assay Procedure.*

Fresh, intact beef thyroids were obtained from a nearby slaughterhouse[3]. The glands were removed 10 to 20 minutes after the animal's death, wrapped in parafilm, and surrounded by ice[4] during the fifteen-minute transit to the laboratory. A single gland was dissected free of the surrounding fat and connective tissue capsule and cut into blocks appropriate for slicing. Slices (40–80 mg.) were made with a manual Stadie-Riggs type microtome. Although all initial work was done with slices 0.45 mm. thick and 2 cm. in diameter, more recent experiments revealed that slices 0.5 or 0.7 mm. thick and 1 cm. in diameter were more uniform in weight and response (see Section H). In summer months, the glands occasionally were so small as to limit the number of slices obtained from one gland to as few as 60, but during the rest of the year 100 to 200 slices could be expected from one gland. The sex of the animals was not found to affect suitability for this assay, but heifers' thyroids were usually too small to provide a sufficient number of

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[2] Fellow of the National Foundation for Infantile Paralysis.

[3] We are grateful for the generous cooperation of Mr. Hugh J. Brom, Plant Superintendent, Seattle Packing Company.

[4] Because most ice taken directly from an ice machine is below 0° C, care was taken to be sure it was melting in order to avoid slight freezing of the tissue, which was found to abolish the response to TSH.

slices. Tissue preparation and slicing were carried out through arm ports inside a refrigerated box kept at 4° C and 100% relative humidity with a spinning disc humidifier. The slices were selected for their freedom from connective tissue and placed on a palette of filter paper which was moistened with medium (described below). They were rinsed collectively three times in 200 ml. of medium at 4° C for a total of 10 minutes to remove blood and easily detached tissue fragments, returned to a fresh filter-paper palette, and allowed to equilibrate at 4° C for 15 minutes to stabilize the increase in weight due to hydration. The individual slices were then rapidly weighed on a torsion balance accurate to 0.2 mg. and returned to numbered positions on a moist filter-paper palette in the cold-moist box, where they remained while medium was pipetted into the incubation beakers.

All slices were rinsed and incubated in freshly prepared Krebs-Ringer-phosphate buffer (2)[5] at pH 7.4. The medium[6] also contained potassium penicillin G (10^{-3}M), dihydrostreptomycin sulfate (6.8×10^{-5}M), and polymyxin B (30 μg. base/ml.). These antibiotics were necessary to permit prolonged incubation without bacterial growth, as tested by smear and culture. The volume of medium in each beaker was proportional to the weight of the individual slice and was kept close to 4 ml. per beaker. This choice of volume is based upon results described below. Standard solutions were prepared by dilution from a more concentrated solution of standard TSH powder[7], kept in the frozen state. The TSH was not added individually to each appropriate beaker, but was included in the preparation of the entire aliquot of medium for replicate beakers. Thus, for each group the TSH concentration in the medium was uniform, and the TSH dose in each beaker was proportional to the weight of the thyroid slice. We have routinely recorded the dose of TSH in U.S.P. milliunits (m.u.) per ml. of incubation medium.

After the slices were rinsed and weighed they were incubated, under air saturated with water vapor, for 21 hours at 38° C in shaking metabolic incubators of the Dubnoff type (3), each designed to carry sixty-five 20 ml. beakers. The shaking rate was 120 strokes per minute; the stroke was 5 cm.

At the end of incubation each slice was gently removed with forceps, spread on dry hard filter paper, turned 3 to 5 times (for a total of 3 to 5 seconds) until no more moisture appeared on the paper, and then rapidly weighed on the torsion balance.

All response data are presented as final weight per 100 mg. initial weight[8]. This parameter is denoted simply as "final weight" hereafter in the text and figures. Because bacterial growth or wide pH shifts impaired the response to TSH, the incubation medium at the end of incubation was routinely checked for bacterial growth and pH; the latter usually declined less than 0.2.

Histological studies were performed according to the "histoquantitative" method of Uotila and Kannas (4). QO_2 measurements were made in the usual way (5) in a Warburg apparatus.

B. *Experimental Design and Statistical Analysis*

The experimental design and method of statistical analysis followed Bliss (6). All experiments adhered to symmetrical design among curves, equal numbers in groups,

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[5] The calcium concentration was reduced to 1.28×10^{-3}M., one-half the usual concentration, to avoid precipitation.

[6] The medium contained 10^{-3}M propylthiouracil (PTU) in many experiments, but later (see below) results showed no effect of this concentration of PTU.

[7] Armour 317–51, 0.8 U.S.P. unit/mg.

[8] It was determined that precision was not increased by presenting the data in terms of initial or final dry weight (determined by drying to a constant weight at 50° C in a vacuum oven) or as a per cent weight increment of the TSH group over the control group.

and equal logarithmic intervals of doses, always estimating the potency of an unknown by comparing the response curve with that of the TSH standard preparation determined simultaneously. Similarly, all effects of changes in the medium, preliminary treatment, slice size, volume, etc., were evaluated by comparison between simultaneous parallel curves. All of the thyroid slices used in one day were cut from the same gland. The response, final weight, was plotted against log dose, and only the linear part of the curve was used. For maximum precision, the corresponding parts of unknown and standard curves which were closely similar in response levels were used (cf. 7, p. 108 ff.). Among the criteria for validity required of each assay were: highly significant regression, absence of significant departure from linearity for each curve, and absence of significant heterogeneity of slope among curves. The index of precision, λ, was calculated as composite error/slope, where the composite error took into account both departure of group means from the line and deviation of individual values from their respective group means (i.e., random sampling error). The 95% ($P=0.05$) criterion of significance was used regularly, both in tests of validity and in estimates of precision. True fiducial limits of the log ratio of potency were calculated from the formula of Bliss (6, p. 508).

Groups of 5 were used in exploratory studies, while definitive experiments were based upon 8 or 10 per group. The standard curve consisted of a control group and four or more dose levels, to span the usual useful dose-response range, and unknowns were run at corresponding points. At least 3 points in the straight-line range were used for calculation, affording a test of linearity and more certain check of slope than that obtainable with two points. Occasionally a "2×2" assay was used when qualitative similarity of dose-response curves had been established and/or the two-point slopes were homogeneous and the response levels closely similar (7, p. 108 ff.). Three-fold dose spacing was usually employed.

Throughout this report the data are presented as mean ± standard error of the mean. In the figures standard errors for points are depicted by vertical bars.

EXPERIMENTAL RESULTS

A. *Relation of Response to the Duration of Incubation*

When slices were removed and weighed at intervals during incubation, it was found that during the first 3 hours their weights remained relatively constant. Figure 1 shows that during the subsequent hours there was a decline in the weight of the control slices. The slices in medium containing TSH lost weight less rapidly, and a sufficient dose of TSH caused the weight to remain steady or even increase after ten hours. The difference between control and TSH-stimulated groups increased progressively with time. An incubation period of 21 hours was chosen for convenience. The final dry weights of the slices were the same in the presence or absence of TSH (1). During the incubation for 21 hours, the dry weight fell to 75.2 ±1.42% of the initial value (which was 22.87 mg./100 mg. wet weight).

Because of the long incubation period, the QO_2 was determined at intervals during two 18-beaker experiments lasting 26 hours. The oxygen consumption of control slices fell steadily from an initial level of 0.42 ±0.04 µl. O_2/hr./mg. wet weight to 0.18 ±0.01 (Fig. 1). In a second experiment the final QO_2 was found to be higher in slices incubated in 2.0 m.u. TSH /ml. than in those incubated in medium alone (0.201 ±0.01 vs. 0.130 ±0.01). However, QO_2 measurements did not lend themselves to the large

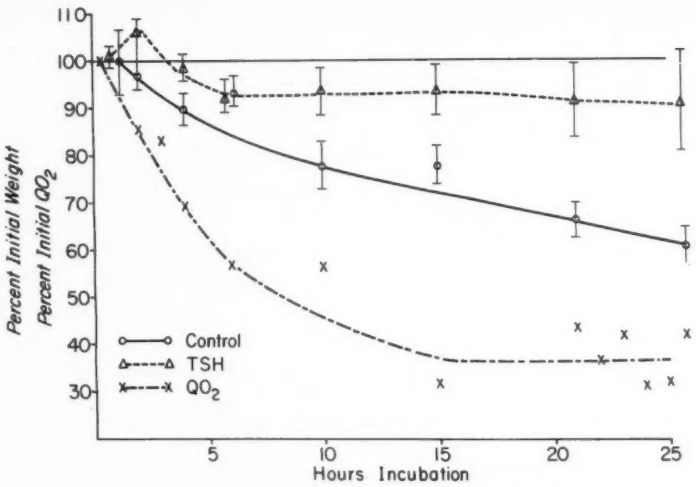

FIG. 1. A time study of the weight and QO_2 of slices during 26 hours of incubation. Each weight is the mean ± standard error of a group of 4. The $Q\curvearrowright_2$, in per cent of initial value, is the mean of a group of 6; only the control group is shown since the TSH group did not differ significantly from this. TSH dose: 0.4 m.u./ml.

numbers of replicate beakers necessary for bioassay, and, as seen in previous experiments (8), the response was not sensitive to small doses of TSH.

B. *Histological Changes*

Histological examination of slices failed to reveal any obvious change in appearance after incubation. Measurements (4) of the follicular components showed that the percentage of colloid, which averaged 74.7 ±0.52 in control slices, was increased progressively in slices stimulated by increasing doses of TSH within the usual dosage range (Fig. 2).

C. *The Influence of Tissue Storage*

There was usually a delay of one to three hours between obtaining the tissue and starting the incubation. However, storing separate slices, exposed to air saturated with moisture, in the cold box at 4° C for three and five hours did not decrease the response below that obtained with fresh tissue; storing slices in piles reduced their responsiveness. The response to TSH was abolished on three occasions when the slices were inadvertently permitted to remain in the incubator at 38° for 15–30 minutes before starting the shaking motor.

D. *The Effect of the Volume of the Medium*

Use of volumes of medium below 2 or above 9 ml. per beaker reduced precision and response, whether referred to absolute dose or concentration of TSH. The effect of volume was tested, eliminating any difference in

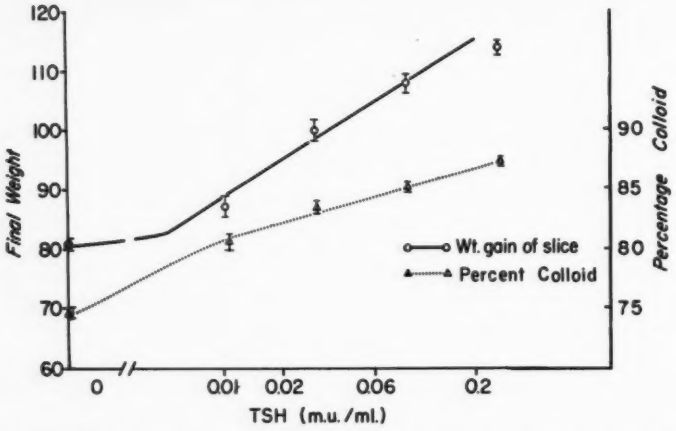

FIG. 2. The TSH dose-response curve of final weight per 100 mg. initial weight (hereafter denoted simply as "final weight") compared with the increase in per cent colloid (follicular volume), measured on the same slices. Groups of 8 slices per point and six histoquantitative measurements per slice. Note different scales.

concentration or total dose of TSH: each beaker contained 9–10 ml. and 3 slices in one curve, 3–4 ml. and 1 slice in the other curve. Fig. 3 shows the poor response in the larger volume. Dye mixing studies showed slower mixing above 4.5 ml.

E. *The Effect of Tonicity, Ca^{++} Concentration, pH, and Enrichment of the Medium*

When the tonicity of the medium was increased to 130 and 170% isotonic, the final weight of the control group was increased and the TSH re-

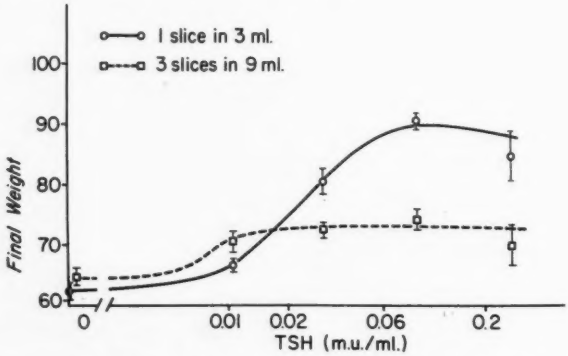

FIG. 3. Comparison between one slice per beaker and three slices per beaker, both in 9 ml. of medium/100 mg. of tissue. Groups of 5 per point.

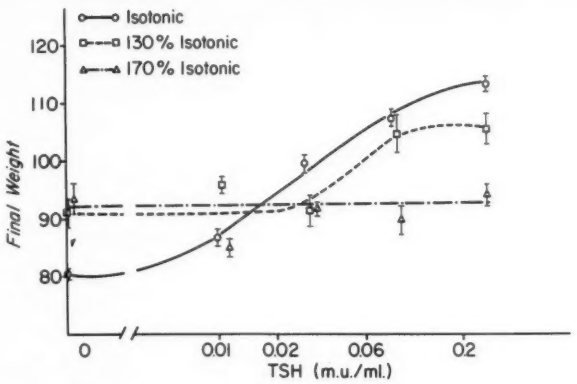

FIG. 4. The response to TSH in hypertonic medium. Groups of 8 per point.

sponse depressed (Fig. 4). When the tonicity was lowered to 50–80% isotonic, no advantage over isotonicity was observed. Use of medium without calcium significantly reduced both the control weight and the slope of response to TSH (Fig. 5). Reducing the pH of the medium to 7.0 had no effect on the assay response, but incubation in medium of pH 6.6 or 6.2 appeared to depress the response slightly, and elevation to pH 8.5 abolished the TSH effect, presumably because of the evident precipitation of most of the calcium. Subsequent experiments used isotonic medium of pH 7.4.

Attempts were made to enrich or alter the medium to facilitate the response or reduce its variability. TRIS[9] did not depress the control weight but completely abolished the response to TSH. Enrichment of the medium with inosine ($5\times10^{-4}M$), with embryo extract[10], or with cortisol (2 μg./ml.) failed to alter the standard response curve significantly. Glucose ($1.1\times10^{-2}M$) added to the medium alone or with a combination of pyruvate ($10^{-2}M$), glutamate ($10^{-2}M$), and adenosine ($10^{-2}M$) depressed both the control value and the response to TSH.

Incubation under pure oxygen or 5% CO_2-95% O_2, with bicarbonate instead of phosphate buffer, failed to improve the bioassay response obtained under air.

F. *The Effect of Potentially Inhibitory Procedures or Substances*

Groups of thyroid slices were heated 10 minutes, during continuous swirling in the usual buffer medium, at three temperatures before use in comparison with slices handled in the usual way (Fig. 6). Slices heated at 70° C. neither responded to TSH nor lost weight during the subsequent incubation for 21 hours. Slices heated at 60° C gave no response to TSH

[9] TRIS, (tris(hydroxymethyl)aminomethane), 3 Gm./1. plus HCl to pH 7.4.

[10] Difco TC Bovine Embryo Extract EE100, diluted 1:50 in the medium.

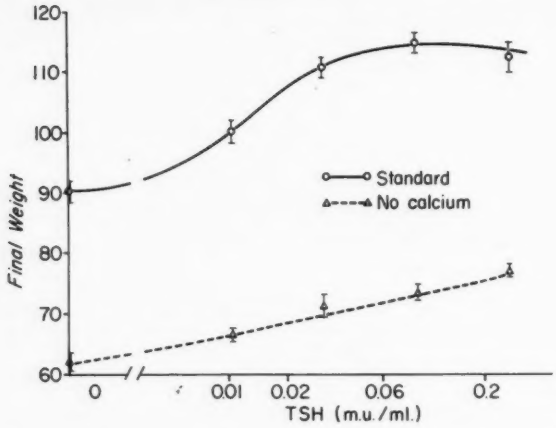

FIG. 5. The effect of omitting calcium from the incubation medium. Groups of 10 per point.

but lost some weight during the 21-hour incubation, although final weights were significantly above those in the standard control group. The slices heated at 50° showed very little rseponse to TSH, but final control weights were identical with those of the standard control group. Preliminary quick-freezing of the tissue on dry ice, or the addition of dinitrophenol ($10^{-3}M$), cyanide ($10^{-3}M$), or mercuric ion ($10^{-5}M$) also abolished the effect of TSH.

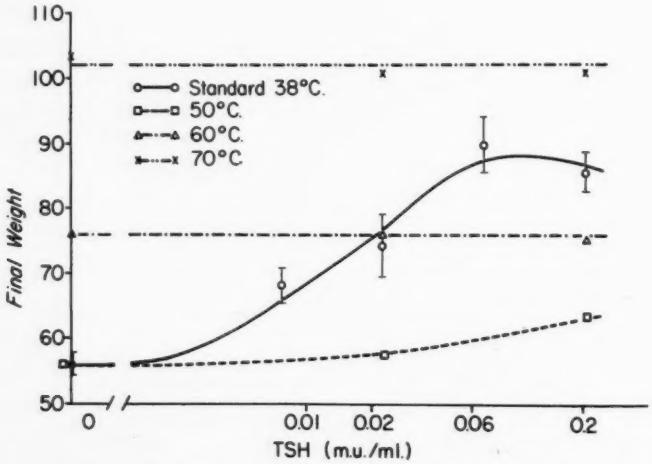

FIG. 6. The response of slices heated for 10 minutes at 70°, 60°, and 50° C before use. The slices used in the standard curve were rinsed for a similar period in an identical manner at 38° C. Groups of 5 per point.

By contrast, fluoride ($10^{-3}M$), thiocyanate ($10^{-3}M$), or propylthiouracil (PTU) ($10^{-3}M$) did not alter the control weight or response to TSH. There was no significant effect of iodide ion in concentrations up to $8\times10^{-5}M$. When the iodide concentration was increased to $8\times10^{-3}M$ a significant increase of 12% was noted in the weight of the controls, and the response to TSH was impaired slightly. Both of these changes were also seen in hypertonic medium, as noted above.

There was no significant difference between a standard curve and a simultaneous curve performed with medium containing 0.4 μg. l-triiodothyronine per ml. Similar experiments were performed with l-thyroxine in concentrations of 0.5, 1, 2, 4, 8, and 16 μg./ml. and with fresh beef thyroid homogenates in a final concentration of 2 mg./ml. (wet weight) without interfering with the standard response to TSH.

Homogenates of beef hypothalamus and liver, kidney, and brain did not interfere with the standard response to TSH.

G. *Specificity of the Response to TSH*

Because of the empirical nature of this bioassay, the establishment of specificity was of fundamental importance. This was undertaken in four ways: (a) establishing that TSH did not alter the change in weight of slices made from other beef tissues, (b) testing a wide variety of substances for TSH-like effect on the thyroid slice weight change, (c) testing TSH which had been inactivated by $KMnO_4$ or by iodine and reactivated by PTU in the manner described by Albert *et al.* (9, 10, 11), and (d) testing several purified and crude TSH or pituitary preparations from different sources to establish similarity of the log dose-response curves.

a. As previously reported (1), TSH was unable to alter the change in weight of other beef tissues, including heart muscle, liver, kidney and anterior hypophysis.

b. Also as described above, a wide variety of substances, including substrates, embryo juice, cortisol, iodide, thyroxine, triiodothyronine and thyroid homogenate failed to produce a TSH-like effect. No TSH response was demonstrated in 0.5 μg./ml. of a lyophilized pituitary substance[11], T-88, known to be free of TSH activity when injected into hypophysectomized rats in a dose of 5 milligrams, or in 0.1, 1.0, or 10 μg./ml. of purified egg albumin, or in fresh homogenates of beef and rat liver, kidney, hindbrain, or midbrain in doses of 20 μg./ml., or in 2 μg./ml. of ACTH[12], i.e., 100 times the amount of TSH powder which simultaneously produced a 50% response. FSH[13], 0.5 μg./ml., produced a response equivalent to 0.01 m.u. of TSH per ml., a reasonable estimate of the known contamination of

[11] Kindly supplied by Dr. I. Gordon Fels, Institute of Experimental Biology, University of California.

[12] Armour "Acthar," K54707.

[13] Purified FSH powder, Parke, Davis and Co., X-5766.

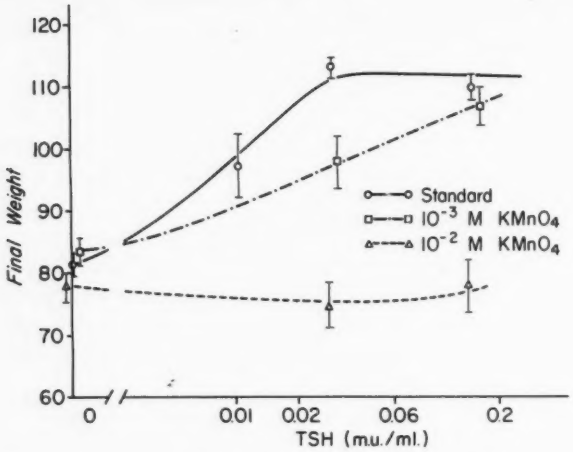

FIG. 7. The response curves of $KMnO_4$-inactivated TSH. 1 mg. standard TSH was incubated one hour at 38° C in 1.1 ml. of buffer containing $KMnO_4$ of the concentration given. Groups of 5 per point.

this preparation of FSH with TSH. 75B, a pituitary fraction[14] rich in exophthalmos-producing substance (EPS) and relatively poor in TSH, at a dose of 0.5 μg./ml. produced a response significantly less than that to 0.05 μg. (0.04 m.u.) of standard (Armour) TSH per ml., while $224S_1$, a fraction[14] rich in TSH, at a dose of 0.024/μg. per ml. produced a maximal response in our assay.

A growth hormone reference standard preparation[15] assayed 1.2%, with fiducial limits 0.6 to 2.4%, as potent as standard (Armour) TSH. A prolactin reference standard preparation[16] produced no significant effect at 0.2 to 20 μg./ml., while the highest dose tested, 200 μg./ml., produced a minimal significant response, equal to that from 0.008 m.u. TSH/ml. and far less than that from 0.024 m.u. TSH/ml.

c. Several solutions of 1 ml. of 1 mg. (800 m.u.) of TSH per ml. of buffer were incubated one hour at 38° C after addition of 0.1 ml. of $KMnO_4$ solution, to produce final concentrations of 10^{-2} to $10^{-6}M$. The resulting solutions were then diluted 100-fold or more with the buffer medium prior to assay. Control solutions were prepared similarly, but without the TSH, and were not significantly and systematically different from the controls

[14] Kindly supplied by Dr. Brown M. Dobyns, Western Reserve University School of Medicine.

[15] Specially purified bovine growth hormone, Somar-A (List 916), supplied by Dr. A. E. Wilhelmi, Emory University, for the Endocrinology Study Section, National Institutes of Health.

[16] Specially purified ovine prolactin, Panlitar (List 806), supplied by Dr. A. E. Wilhelmi for the Endocrinology Study Section, N.I.H.

without $KMnO_4$. As indicated in Fig. 7, TSH was inactivated completely ($\geq 80\%$) in $10^{-2}M$ $KMnO_4$ and partially in $10^{-3}M$ $KMnO_4$.

Using conditions expected to produce complete inactivation of TSH (9), 4 ml. of a solution of 0.4 mg. (300 m.u.) of TSH per ml. was mixed with 1 ml. of a 1:5 dilution of Lugol's solution and allowed to stand two hours at room temperature. The final turbid mixture was diluted to 10 ml. with water. An aliquot was diluted with medium for assay, while 0.05*M* PTU was added dropwise to the remainder to decolorization and formation of a clear solution (1–3 drops excess). TSH was found to be completely inactivated (≥ 96–99% in four experiments) by the I_2 treatment and reactivated (67%, with fiducial limits 34–126%, and 109%, with fiducial limits 68–186%, in two experiments) by the PTU.

d. Figure 8 shows the closely similar assay curves obtained from four different purified TSH preparations, all of bovine origin except for Parke-Davis $50P_4$, which was from hogs and sheep. Figure 9 shows the parallelism between the curve for purified TSH and that obtained with various doses of beef and rat pituitary homogenates, and Figure 10 shows comparison of bovine and human pituitary homogenates. No significant differences in slope or range were noted.

H. *Statistical Results*

Disqualification of assays for heterogeneity of slope among component curves did not occur. No trend of variance with dose level was observed. Significant departure from linearity, except at the unused maximal or minimal ends of the curve, was very infrequent. Variation in response and

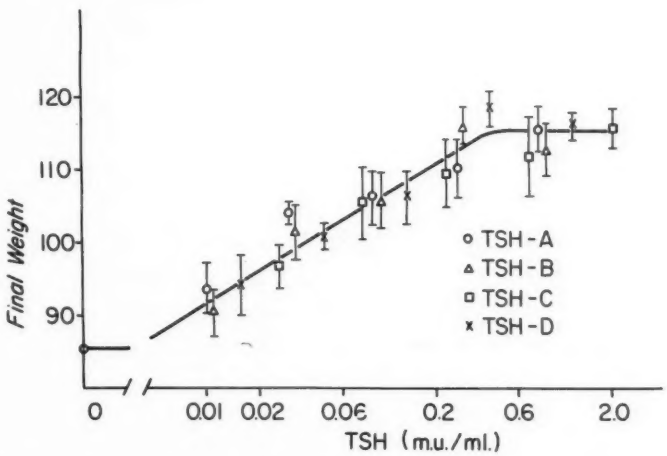

FIG. 8. The response of four different purified preparations of TSH: "A" is Armour #317-147; "B" is Armour #317-51; "C" is Parke, Davis 50P4; "D" is Armour "Thytropar" Lot P2303. Groups of 5 per point.

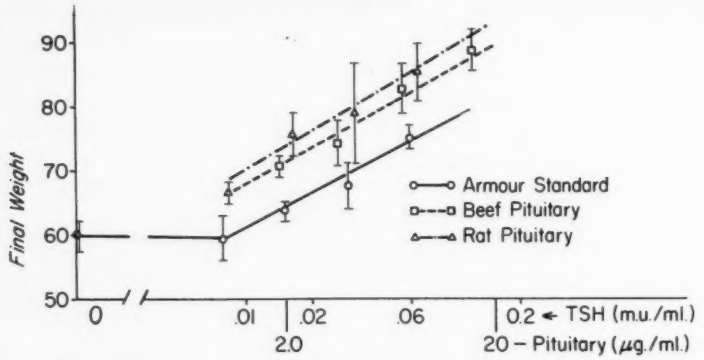

FIG. 9. The response curves from dilutions of rat and beef pituitary homogenates compared with the standard purified TSH. Groups of 5 per point.

"baseline" (control values) among days was great compared to that between curves run on the same day. The impression that this variation was due to differences among animals was supported by results of standard curves obtained on four different glands simultaneously. The four curves differed in slope in a manner similar to the variation in slope and error in different experiments, which is seen in the various figures in this report.

Results from forty representative single curves obtained in the spring of 1956, using slices 2 cm. in diameter and 0.45 mm. thick, are summarized as follows: The minimum (statistically significant) detectable concentration of TSH varied from $\leq$0.002 m.u. to 0.03 m.u./ml., with a median of 0.01; 75 per cent of the values were 0.01 or less. The dose range of linear response varied from 3- to 81-fold, with a median range of $\geq$9-fold; 80 per cent of the values were equal to or greater than 8- or 9-fold, permitting at

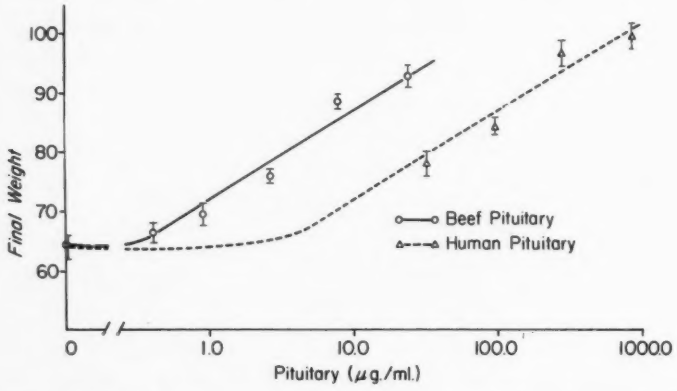

FIG. 10. The response to various dilutions of human and beef pituitary homogenates. Groups of 5 per point.

least three usable points at the usual 3-fold dose spacing. Thus, since then our standard curve has usually had the following dose levels: control, 0.008, 0.024, 0.072 and 0.22 m.u./ml.

Although the index of precision, λ, for the curves mentioned above ranged from 0.20 to 0.88, with a median of 0.40, better results have been obtained since the use of slices 1 cm. in diameter and 0.5 to 0.7 mm. thick. Forty-one consecutive curves, using the latter slice size, have evinced a λ of 0.08 to 0.65, with a median of 0.28. Their slope, b, varied from 9.5 to 42.4, with a median of 21.3.

DISCUSSION

Results of assays for TSH were reviewed comprehensively in 1952 (12) and, in part, more recently (13, 14). Except for methods published in the last few years, rigorous comparison of published data is precluded by several factors: lack of common or comparable standard preparations, failure to use experimental design affording optimal precision, even the use of fixed response units without simultaneous determination of a standard curve with each assay, and insufficient presentation or analysis of data (11). It might be estimated that the best earlier methods of TSH assay (e.g., 15) probably offer the same order of precision as the more recent ones (see below). Some comparisons, chiefly with regard to sensitivity, have been derived from the literature (11, 12, 13, 14, 16). In general, the classical gravimetric, histological, and chemical methods of measuring thyroid response to thyrotropin have proved useful in gross studies of pituitary TSH content and in fractionation of extracts but are of such limited sensitivity that they would probably not be applicable to measuring TSH in biological fluids. There are two exceptions: D'Angelo and coworkers' "stasis tadpole" method, in its several modifications (17, 18, 19, 20), was reported to exhibit a minimum stimulating dose of 0.085 μg. of a preparation equivalent to ca. 0.2 U.S.P. milliunits (m.u.)[17], and DeRobertis' (21, 22, 26) method of counting intracellular droplets in the guinea pig thyroid detected a minimal dose of 0.0002 J.S. units (ca. 0.02 U.S.P. m.u.[18]). The droplet method was found to respond to nonspecific stimuli, according to Rawson (quoted by Loraine, 23) and Dvoskin (24, 25, 26) in his modification of DeRobertis' procedure. Neithre DeRobertis nor D'Angelo reported using an assay which included parallel standard determinations; single unknown points were interpolated on a fixed standard curve. Hence their precision cannot be determined from the published

[17] This comparison is based on personal communications from Mr. Wayne Donaldson, of Parke, Davis and Co., that Parke, Davis preparation #09993 contains about 2 U.S.P. units per mg.

[18] Estimates here and subsequently in the paper are based on a personal communication from Armour Laboratories stating that 1 U.S.P. unit is equivalent to about 10 J.S. units.

data. However, recent information from D'Angelo (27) has indicated that the precision of his tadpole assay at present compares favorably with that of other methods.

Refinements of histological methods have been devised recently (4, 12, 28). Several of these "histoquantitative" parameters have been compared directly (12, 28, 29, 30) and found to afford similar sensitivity and precision[19]. All of these methods are limited in their promptness and convenience by the necessary histological preparation.

The other principal development, reviewed by Lamberg in 1955 (14), has been the use of radioisotopes in chicks, guinea pigs, or rats by measuring I^{131} release or uptake of P^{32} uptake. Lamberg (13) presented complete data and a statistical analysis of his results with P^{32} uptake by the chick thyroid, which he found comparable in sensitivity and precision to the "histoquantitative" methods, as follows: within the range 0.005–0.05 J.S. units (ca. 0.5–5.0 m.u.) "a dose can as a rule be distinguished from one four times as large."[20] He used 7 chicks per group and three groups per curve plus one control group; this total of 49 animals per day constituted both a "practical maximum limit" and also almost the minimum number permitting adequate precision. Since Lamberg's review, reports of several assays utilizing radioactive isotopes have appeared, including brief statistical analysis and standards which can be compared directly in terms of U.S.P. m.u. Levey, Cheever, and Roberts (32) measured the 4-hour I^{131} uptake in rats 20 hours after a single injection of TSH. They compared rats pretreated by injection of thyroxine or thyroxine plus propylthiouracil, feeding of thyroid powder, or hypophysectomy. Rats hypophysectomized or prefed thyroid powder were the most sensitive. The former had an effective dosage range of 0.625 to 2.5 m.u., with a λ of 0.24 (the number of assays on which this was based was not given), while the latter afforded more convenience with a range of 2.5–160 m.u. (λ was not stated). Greenspan *et al.* (33) modified the chick thyroid P^{32} uptake method by using one intracardiac injection of TSH and utilizing, as the response metameter, the square root of the time to obtain 1024 counts in the thyroid. The range of usable response was 0.25 to 5 milliunits; an average λ of 0.34 was obtained over several months. They used three groups of 5 chicks at a three-fold dose spacing. About 50 chicks per day could be used in an assay. Preliminary reports by Morton and Schwartz (34) and Florsheim, Moskowitz, and Morton (35) described measurement of the incorporation of

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[19] Tala (12) reported the great sensitivity of 0.00001 J.S. units by use of the percentage of epithelium parameter in guinea pigs, but the work of Lamberg in the chick (13) and Tala *et al.* (31) in the guinea pig later demonstrated that the usable, approximately linear, assay range began at 0.005 to 0.01 J.S. units (ca. 0.5 to 1.0 U.S.P. m.u.).

[20] Using $P = 0.05$ as the criterion of significance.

P^{32} into a lipid fraction of thyroid slices. The linear range was 2 to 100 m.u. Specificity was established, as with other P^{32} methods, but precision was not estimated.

The assay method described in this report may be reviewed in terms of the factors which determine the usefulness of any bioassay: specificity, sensitivity, usable (linear) range, inherent precision (reflected in λ), and economy, especially of time. Its specificity was established. The usual minimum stimulating dose of ≤ 0.01 m.u. per ml. medium is closely comparable to the sensitivity reported by DeRobertis (21, 22) and is more sensitive than other reported methods. The dose range of linear response and precision are of the same order as the other methods which present sufficient data or analysis to permit comparison. The median λ of 0.28 is comparable to that reported by Levey and coworkers (32) or Greenspan *et al.* (33). The assay utilizing beef thyroid slices excels other reported methods in economy, especially of time.

This assay is unusual in utilizing a response for which there is no known counterpart *in vivo*. The biological significance of the increased water gain of thyroid slices incubated in the presence of TSH is not clear at present. It must be independent of known functions of the thyroid gland involving iodine metabolism, since it was not altered by propylthiouracil, thiocyanate, or relatively high concentrations of organic or inorganic iodine compounds in the incubation medium.

The response was blocked by cyanide or dinitrophenol and by brief freezing or incubation 10 minutes at 50° C; it was also abolished by short periods (ca. 15 minutes) of incubation at 38° C without shaking or mixing. These results support the concept that an oxidative process and a heat-sensitive enzyme system are essential for the weight response. The final dry weights of control and TSH-stimulated groups did not differ. Thus the weight change appears to be due primarily to entry of the medium fluid into the slice. Under the dissecting microscope, the follicles of slices incubated for 21 hours appeared markedly swollen and tense, discharging their contents when pricked with a needle. Histological examination showed a greater mean follicle diameter and percentage of colloid in the slices incubated with TSH than in the control group. Since the follicles comprise most of the slice volume the swelling of the follicles may account for the weight gain. A possible reason for this would be a change in the colligative properties of the colloid. DeRobertis (36, 37) observed that TSH stimulated proteolytic enzyme activity in the thyroid follicle *in vivo*, a response not inhibited by PTU or thiocyanate. It is possible that, in response to TSH, hydrolysis of large molecules of thyroglobulin within the thyroid follicle occurs *in vitro* and the resultant increase in osmotic pressure causes imbibition of water. Further studies of physical and chemical properties of the colloid are in progress.

SUMMARY

A new method for the bioassay of thyrotropic hormone (TSH), based on the weight response of beef thyroid slices incubated 21 hours at 38° C in a shaking incubator, is presented. TSH induced higher final weights compared with the control group.

Histoquantitative evidence is presented that the response consisted of swelling of the follicles, due to the entry of fluid from the incubation medium.

The response was blocked by preheating the slice for 10 minutes at 50° C, or by the presence of dilute concentrations of dinitrophenol, cyanide, or mercuric ion, but was not blocked by fluoride, thiocyanate, propylthiouracil, or iodide.

The assay was shown to be specific; of adequate range; sensitive to ≤ 0.01 U.S.P. milliunits of TSH/ml.; as precise as other reported current methods, with a median λ of 0.28; and unusually simple and economical.

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A CHEMICAL AND HISTOCHEMICAL STUDY OF ALIESTERASE IN MOUSE ADRENAL CORTEX UNDER HORMONAL INFLUENCES AND ALTERED SODIUM INTAKE[1,2]

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MARKED changes in the distribution of histochemically determined alkaline phosphatase in the mouse adrenal cortex under different hormonal conditions have been demonstrated (1). The locus of this enzyme is disparate following administration of testosterone proprionate, estradiol benzoate or adrenocorticotrophic hormone (ACTH) to castrate animals. In view of these topographic alterations obtained in the case of alkaline phosphatase it appeared desirable to investigate the effect of varied physiological status on other enzymes in this tissue. The behavior of chemically and histochemically determined non-specific esterase (aliesterase) under hormonal influences and altered sodium intake forms the subject of this paper.

METHODS

All work was carried out on adrenal glands from pure bred BALB/c Jax male mice 90–180 days of age. Aliesterase was determined histochemically as described by Pearse (2) using alpha-naphthyl acetate as substrate and Fast Blue Salt B (General Dyestuff Company), Diazo Garnet GBC (duPont Company) or Diazo Red RC (duPont Company) as coupling agents. Diazo Garnet GBC produced the most satisfactory preparations. Adrenals for study were fixed in 10% formalin buffered to pH 7 and made isotonic with a phosphate buffer system. Fixation was carried out for 12 hours at 4° C. Tissues were sectioned at 15–20μ on a freezing microtome and dry-mounted on slides. Prior to immersion in the histochemical substrate, sections were extracted for one minute in acetone at room temperature to remove neutral fats. This procedure did not alter the intensity or the distribution of dye deposition in sections. These sections were incubated in the substrate solution with constant agitation for 10 minutes at 20° C. Sections treated with water at 90° C for 10 minutes served as controls. Histochemical determinations were made on a minimum of 5 animals from each treatment group.

Quantitative determinations of aliesterase activity were carried out on homogenates of fresh adrenal tissue following the procedure of Gomori (3) and employing alpha-naphthyl acetate as substrate and Fast Red Salt ITR (General Dyestuff Company) as coupling agent. The substrate solution for quantitative determinations contained 10^{-5} *M* eserine sulfate to block the activity of cholinesterase known to be present in the medullary tissue of these glands (Allen, unpublished data). This concentration of eserine had no

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[2] Steroid hormones were furnished through courtesy of the Schering Corporation.

effect on the amount or distribution of dye deposition in the cortex when checked in histochemical preparations. Incubation was carried out at 37° C for one hour. Following addition of dye coupler these tubes were allowed to stand against their controls for 15 minutes at room temperature and were then read in a Klett-Summerson colorimeter using a 500–750 mμ filter. Total nitrogen in the samples used for quantitative analysis was determined by a micro-Kjeldahl method (4).

RESULTS

In normal control animals histochemical preparations show esterase activity to be present in the cytoplasm of all cortical epithelial cells (Fig. 1). Nuclei and connective tissue cells show little or no activity. Enzyme activity in the zona glomerulosa is distinctly greater than that seen in the zona fasciculata. In all zones of the cortex certain cell groups show local differences of enzyme activity.

Following maintenance of animals on a low sodium diet (Nutritional Biochemicals Corporation) and distilled water for a period of 4 weeks a marked change in the activity of aliesterase is noted in histochemical preparations (Fig. 2). Comparison of the zona glomerulosa of normal and treated animals shows in the latter group an increase in the amount of dye deposition. Aliesterase activity in the zona fasciculata appears unchanged from normal although an occasional animal will show a slight increase in dye deposition in this area. Quantitative determinations of enzyme activity appear to substantiate the histochemical observations (Table 1); thus highly significant increases ($P < 0.001$) in both total activity as well as in specific activity of aliesterase are noted.

Administration of 0.3 *M* NaCl in the drinking water for a period of 4

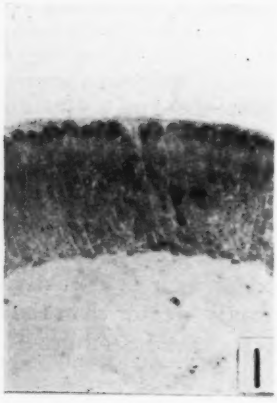
TABLE 1. QUANTITATIVE ESTIMATION OF ALIESTERASE ACTIVITY IN MOUSE ADRENAL CORTEX*

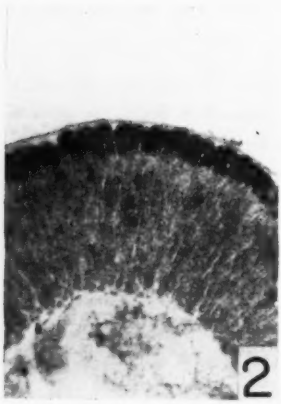
Treatment	Number of animals	Mean log total activity†	Mean log specific activity‡
Normal Control - Sodium-free diet, 4 weeks	20	1.815 ±0.186 (—)	0.284 ±0.185 (—)
200 μ DCA daily, 4 weeks	7	2.513 ±0.148 (<0.001)	1.486 ±0.230 (<0.001)
200 μ DCA daily, 8 weeks	5	2.544 ±0.195 (<0.001)	0.864 ±0.158 (<0.001)
0.3 *M* NaCl, 4 weeks	9	1.861 ±0.180 (0.6–0.5)	0.272 ±0.118 (0.9–0.8)
0.3 *M* NaCl, 8 weeks	5	2.299 ±0.122 (<0.001)	0.731 ±0.100 (<0.001)
4 IU. ACTH twice daily, 2 weeks	10	1.977 ±0.150 (0.05–0.02)	0.329 ±0.085 (0.5–0.4)
1 IU. ACTH twice daily, 2 weeks	9	2.485 ±0.266 (<0.001)	0.429 ±0.198 (0.05–0.02)
Castrate, 4 weeks	8	2.571 ±0.152 (<0.001)	0.419 ±0.092 (0.05–0.02)
	8	1.779 ±0.255 (0.6–0.5)	0.209 ±0.226 (0.6–0.5)

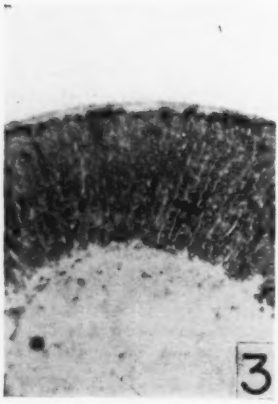
* Activity figures are followed by Standard Deviation with T-test probability values in reference to Normal Control group in parentheses.

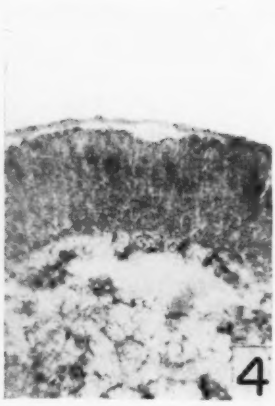
† Calculated amount of alpha-naphthol liberated by a pooled homogenate composed of both adrenal glands.

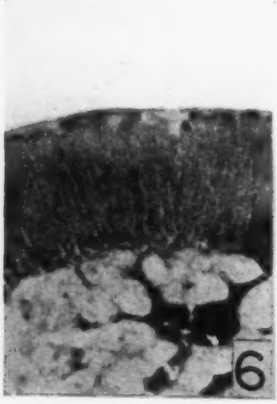
‡ Calculated micrograms of alpha-naphthol liberated per microgram of total Kjeldahl nitrogen in the sample under analysis.

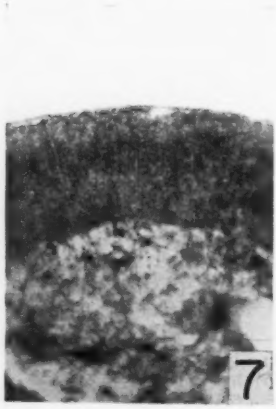












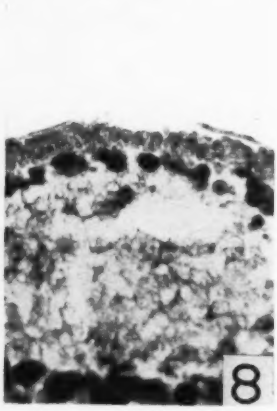


PLATE I

and 8 weeks causes alteration in the histochemical activity of aliesterase in both the zona fasciculata and zona glomerulosa. After treatment for 4 weeks (Fig. 3) there is marked diminution in the amount of dye deposition in the zona glomerulosa as compared with normal control animals. Dye deposition in the zona fasciculata is increased over that seen in the normal control group. Quantitative estimates of both total activity and specific activity (Table 1) show significantly increased levels of enzymatic liberation of alpha-naphthol ($P < 0.001$). Treatment for 8 weeks produces a further depression in histochemically visualized aliesterase activity in the zona glomerulosa (Fig. 4). Activity of the enzyme in the zona fasciculata (Fig. 4), as judged on histochemical preparations, appears below normal. Quantitative estimates (Table 1) of total activity indicate that significantly more ($P = 0.05$–0.02) alpha-naphthol is liberated on this basis than in normal control animals but specific activity estimates are comparable with those obtained from normal control animals ($P = 0.5$–0.4).

All figures show the distribution of aliesterase in formol fixed frozen sections treated with alpha-naphthyl acetate and Diazo Garnet GBC; photographed at 100× using a Wratten 58 filter.

FIG. 1. Distribution of aliesterase in the adrenal cortex of normal male. The zona glomerulosa shows more activity than does the zona fasciculata. Note areas of intense local activity in the zona fasciculata. Nuclei and the cells of connective tissues are negative.

FIG. 2. Distribution of aliesterase in the adrenal cortex of animal maintained on low sodium diet and distilled water for 4 weeks. Note great increase in aliesterase activity in the zona glomerulosa. Only a slight increase in dye deposition in the zona fasciculata is evident.

FIG. 3. Distribution of aliesterase in the adrenal cortex of animal given 0.3 *M* NaCl as drinking water for a period of 4 weeks. Enzymatic activity is depressed in the zona glomerulosa and increased in the zona fasciculata.

FIG. 4. Distribution of aliesterase in the adrenal cortex of animal given 0.3 *M* NaCl as drinking water for a period of 8 weeks. Further decrease in enzymatic activity is noted in the zona glomerulosa and activity in the zona fasciculata is below normal levels (Fig. 1).

FIG. 5. Distribution of aliesterase in the adrenal cortex of animal receiving 4 I.U. ACTH subcutaneously twice daily. Increase in the activity of aliesterase in the zona fasciculata is apparent but the zona glomerulosa is unchanged from normal (Fig. 1).

FIG. 6. Distribution of aliesterase in the adrenal cortex of animal receiving 200 μg. DCA daily for a period of 4 weeks. Activity in the zona glomerulosa is decreased as compared to normal control animals (Fig. 1) while increased enzymatic activity is noted in the zona fasciculata.

FIG. 7. Distribution of aliesterase in the adrenal cortex of animal receiving 200 μg. DCA daily for a period of 8 weeks. Further depression of aliesterase activity is noted in the zona glomerulosa and activity in the zona fasciculata is below normal levels (Fig. 1).

FIG. 8. Distribution of aliesterase in the adrenal cortex of animal receiving 200 μg. cortisone acetate daily for a period of 4 weeks. Enzymatic activity is absent in the zona fasciculata but dye deposition in the zona glomerulosa is comparable to that seen in normal control animals (Fig. 1). Dark pigment in the region of the zona fasciculata represents non-enzymatic staining of areas of brown degeneration.

Subcutaneous administration of 200 μg. of desoxycorticosterone acetate (DCA) (Schering) daily in 0.1 cc. sesame oil for 4 and 8 weeks produces effects similar to those noted following administration of 0.3 *M* NaCl in the drinking water. Following 4 weeks of treatment (Fig. 6) histochemical preparations show a depression of aliesterase activity in the zona glomerulosa and an increase in activity of the zona fasciculata as compared with normal control animals. Eight weeks of treatment (Fig. 7) further depresses aliesterase activity in the zona glomerulosa. Activity in the zona fasciculata (Fig. 7), as judged on histochemical preparations, is below that seen in control animals. Quantitative determinations (Table 1) of both total activity and specific activity following 4 weeks of treatment show significant elevations ($P < 0.001$) over normal. After 8 weeks of treatment aliesterase activity measured on the basis of total activity ($P = 0.6$–0.5) and specific activity ($P = 0.9$–0.8) has returned to normal.

Four International Units (I.U.) of ACTH (Armour) administered subcutaneously twice daily for a period of 14 days to intact animals results in increased dye deposition in the zona fasciculata (Fig. 5). No change is perceptible in the zona glomerulosa of these animals in histochemical preparations. Quantitative determinations (Table 1) of total activity show a significant elevation ($P < 0.001$) as compared to normal control animals; specific activity also is significantly elevated ($P = 0.05$–0.02).

Administration of 1 I.U. ACTH (Armour) subcutaneously twice daily to Intact animals produces histochemical changes identical to those just noted in the preceding paragraph. Quantitative determinations (Table 1) show significantly elevated total activity ($P < 0.001$) and specific activity ($P = 0.05 - 0.02$) as compared to normal controls.

Administration of 200 μg. cortisone acetate (Schering) as an aqueous suspension daily for 4 weeks results in marked changes in the histochemical distribution of aliesterase in the adrenal cortex (Fig. 8). Enzyme activity is abolished in the zona fasciculata; however, the zona glomerulosa appears unchanged from normal. Areas of brown degeneration are prominent in the zona fasciculata of treated animals. The material in such areas reacts with the coupling agents employed to form a dark colored pigment.

Castration for a period of 4 weeks produces no histochemical change in the distribution or activity of aliesterase in the adrenal cortex. Quantitative determinations of total activity and specific activity (Table 1) show no significant differences ($P = 0.6$–0.5) from normal controls.

DISCUSSION

These observations suggest a physiological dichotomy between cells of the zona glomerulosa and cells of the zona fasciculata in the mouse adrenal cortex. The activity of aliesterase in the zona glomerulosa is elevated by conditions of sodium deprivation and depressed by conditions tending to elevate the blood Na/K ratio such as administration of DCA (5) or excess

dietary sodium chloride. Response of the enzyme in the zona fasciculata under identical conditions is quite different. Sodium deprivation produces little effect on the activity of aliesterase in this region but sodium flooding causes an increase in the activity of the enzyme. Increased activity of aliesterase in the zona fasciculata is apparent 4 weeks after institution of treatment but a return to near normal is observed after 8 weeks of treatment.

Further evidence of a physiological difference between the zona glomerulosa and the zona fasciculata in the mouse adrenal cortex may be adduced from behavior of aliesterase in response to exogenous ACTH or cortisone acetate. Administration of ACTH in high and low dose levels produces a significant increase in the activity of aliesterase in the zona fasciculata but causes no perceptible change (histochemical) in activity of the enzyme in cells of the zona glomerulosa. Aliesterase activity in the zona fasciculata is abolished following inhibition of ACTH production by exogenous administration of cortisone acetate (6), while activity (histochemical) of the enzyme in the zona glomerulosa remains unaffected. It should be noted that visual quantification of dye deposits in tissue sections is subject to a considerable error (7). Small but important changes in the activity of zona glomerulosa cells may, therefore, have been imperceptible.

These results are most nearly in accord with the zonal hypothesis of adrenal cortical function as set forth by Swann (8) and elaborated by Greep and Deane (9), and Greep and Jones (10). They suggest that the zona glomerulosa is chiefly responsible for secretion of mineralocorticoids while the zona fasciculata secretes mainly glucocorticoids.

It is unfortunate that a definite physiological role cannot be assigned to aliesterase. The use of alpha-naphthyl acetate as a histochemical and chemical substrate probably yields information concerning enzymatic activity against simple fatty acid esters as well as against fatty acid esters of glycerol (11, 12). Comparison of the results obtained in the present study with alterations in Sudan stainable material (neutral fat) as described by Miller (13) in the mouse adrenal cortex suggests that increases in the activity of aliesterase are correlated with depletion of neutral fat reserves in this tissue. It is tentatively suggested that a physiological role for aliesterase in the mouse adrenal cortex may be the liberation of fatty acid from neutral fat depots.

Other results indicate that the enzymatic response of the adrenal cortex to physiological alteration is variable. In the adrenal of hypophysectomized rats phenolsulfatase activity in the zona fasciculata is depressed (14) while beta-glucuronidase activity in this zone is increased (15). Zweifach *et al.* (16) investigated the capacity of histological sections of rat adrenal cortex to reduce 2,3,5-triphenyl tetrazolium chloride under conditions similar to those employed in the present work. The response of this reductase(s) is similar to that of aliesterase in the mouse adrenal cortex. The independence

of mouse adrenal aliesterase from influence by testicular hormone is in marked contrast to the behavior of epithelial alkaline phosphatase in the same tissue (1). Apparently not all enzymes of the adrenal cortex are concerned with the secretory process.

SUMMARY

The chemical and histochemical activity of aliesterase in the mouse adrenal cortex has been studied under a variety of physiological conditions. Restriction of dietary sodium increases the activity of the enzyme in the zona glomerulosa and has little effect upon aliesterase activity in the zona fasciculata. Administration of desoxycorticosterone acetate by injection or of 0.3 *M* NaCl in the drinking water for 4 and 8 weeks depresses the activity of aliesterase in the zona glomerulosa. Activity of the enzyme in the zona fasciculata under identical conditions is increased at 4 weeks but has returned to normal or nearly so at 8 weeks. Exogenous cortisone acetate abolishes aliesterase activity in the zona fasciculata but has no perceptible effect on the enzyme in the zona glomerulosa. Subcutaneously administered adrenocorticotropic hormone increases the activity of aliesterase in the zona fasciculata but appears to have no effect upon the activity of the enzyme in the zona glomerulosa. These results suggest that the zona glomerulosa of the mouse adrenal cortex is chiefly responsible for secretion of mineralocorticoids while the zona fasciculata secretes mainly glucocorticoids.

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PLASMA LEVEL OF ANTIDIURETIC HORMONE AND SERUM OSMOLARITY IN NORMAL HUMAN ADULTS[1]

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IN THE attempts to determine the conditions under which the antidiuretic hormone (ADH) is secreted, one finds that the inhibition of diuresis is caused by osmotic, haemodynamic and nervous stimuli. Until now the quantity of ADH present in the blood could only be estimated indirectly through its antidiuretic effect. A definite quantitative biological determination was lacking, because of the inadequate sensitivity or low specificity of the experimental animals used, especially the rat. There were also many sources of error in the bioassay of antidiuretic substances in urine. Furthermore, it did not permit an accurate quantitative evaluation of the ADH secretion from the neurohypophysis (1). Conclusions regarding the relation between diuretic inhibition and the ADH activity in plasma are of little significance, since diuresis can be modified actually by changes in the filtration rate, intrarenal haemodynamics, and the electrolyte reabsorption from the renal tubules. The latter are under the control of the adrenal cortex.

Due to the previous difficulties, we have chosen to make our studies with a new, highly sensitive, and specific biological method on the earth toad, *Bufo bufo*. We determined the ADH plasma level of healthy adult human subjects under various conditions of water-salt metabolism.

METHODS

The ADH bioassay was done with fresh, heparinized plasma, drawn from the cubital vein, and administered to sexually mature, non-moulting male toads weighing between 25 and 50 gm. This was carried out according to the procedure given by Buchborn (2). The animals were placed in tap water having a temperature of 17–23° C. eight hours before commencing the experiment in order to bring the toads into equilibrium with the water. Every animal in each group of five toads was injected into the dorsal lymph sac with a quantity of solution equaling 5% of its body weight. The solutions had the following composition: (ADH standard solution)[2] with 100 μU/ml. in 0.154*M* NaCl sol.; a dilution of human plasma and distilled water in the ratio of 1:1; a dilution of human plasma, distilled water and 0.154*M* NaCl sol. in the ratio of 1:1:2. All injected solutions had a 0.154 *M* concentration and were slightly hypotonic to amphibian plasma.

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[1] This work is supported by a grant from the Deutsche Forschungsgemeinschaft.

[2] A highly purified Pitressin preparation, kindly supplied by Prof. du Vigneaud, New York, served as the standard at first. Subsequently a constant batch of commercial product called Tonephin (Hoechst) No. 6/145 was used.

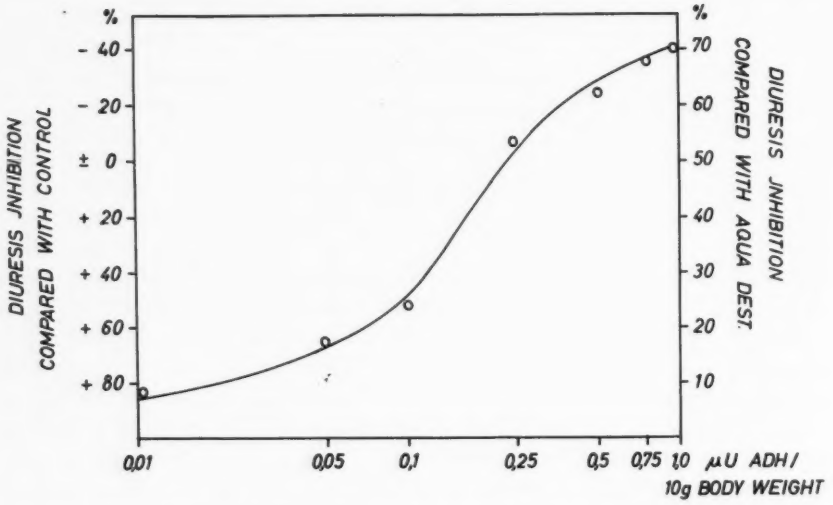

FIG. 1. Dose-response curve for the inhibition of diuresis by ADH on the hydrated toad *Bufo bufo*.

The mean per cent value of diuretic inhibition in each animal group 2 hrs. after injection, in comparison to the mean diuretic value taken three hours before the injection, served as a response index. We were able to plot semilogarithmically an S-shaped dose-response curve with a constant slope between 10 and 1500 µU ADH/ml. distilled water. It can be transposed into a linear course according to Hazen's method (Fig. 1).

The accuracy of the test shows an average precision index of $\gamma = 0.228$ (Gaddum), i.e. the degree of variability is $\pm 20\%$ of the determined single dose. With respect to our observations the diuresis of the toad was neither responsive to ferritin nor to 5-oxytryptamin (serotonin). Likewise the diuresis did not respond to oxytocin, in contrast to other amphibians.

The serum osmolarity of the same human blood sample was measured cryoscopically and the results were expressed in mOsm/L. The sodium determination was made with a Zeiss flame photometer using a wave length of 589 Å. A total of 34 healthy male and female adults were examined. Different conditions of water metabolism were attempted through a) an ingestion of 1500 ml. of fluid, b) i.v. infusion of a hypertonic NaCl solution and c) 2–4 days of thirst with an unrestricted quantity of dry foods.

RESULTS

We found the ADH plasma concentration to be between <10 and 220 µU/ml. plasma. The found values were classified according to the conditions of water intake and the arithmetic mean derived from the ADH values for each of the three groups. One obtains a significant difference in the mean value between Group I and III (Fig. 2). Between Groups I and II, the cases with hydration and normal fluid intake, the difference is statistically uncertain. This is undoubtedly due to the small number of experimental subjects.

In spite of the differences between groups, the individual figures show a great variation about the mean value. This is most pronounced in thirst, and does not have any correlation with the duration of water restriction.

In contrast to this, there is a close relationship between the ADH plasma concentration and the serum sodium concentration. Finally if one seeks a connection between the ADH plasma level and serum osmolarity one finds a significant positive correlation with $P < 0.001$. The plasma ADH concentration decreases proportionally with the exponential fall of serum osmolarity. It is therefore inversely proportional to the log of the serum water content. The regression of the ADH plasma concentration to serum osmolarity is shown in Figure 4. The mathematical equation for this reads

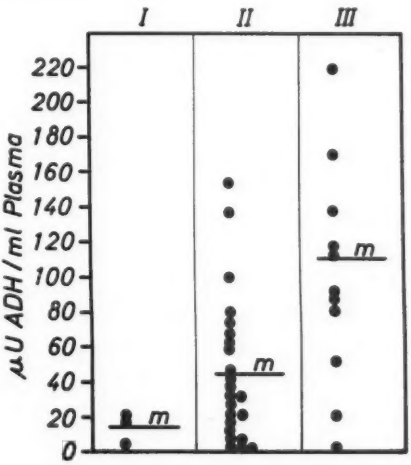

FIG. 2. The ADH plasma concentration following (I) 1500 ml. fluid intake, (II) fluid and food intake *ad libitum*, (III) thirst for 20–75 hours or i.v. infusion of hypertonic NaCl solution.

$Y(\text{in } \mu U \text{ ADH/ml.}) = 0.476x - 3.42$. The variance for this regression is essentially smaller than that for the ADH plasma level and serum sodium concentration. Its significance is correspondingly higher.

DISCUSSION

Up until now difficulties in method have prohibited an accurate determination of physiological quantities of ADH in plasma. A further difficulty arose in that the presence of two substances, which normally appear in serum, namely ferritin (3) and serotonin (4, 5) had an antidiuretic effect on the experimental animals which could not be distinguished from the ADH effect. The ADH determination on the toad is free of this disadvantage and its sensitivity permits the detection of ADH in quantities as small as 10 μU/ml. plasma.

In our normal subjects with unrestricted fluid intake, the ADH values in plasma lie between <10 and 140 μU/ml. This great variation can first be understood, only when one simultaneously measures the serum osmo-

larity and takes into consideration its relationship to the ADH values (Fig. 4). The same relationship exists between the cases with hydration and dehydration. From the close correlation between serum osmolarity

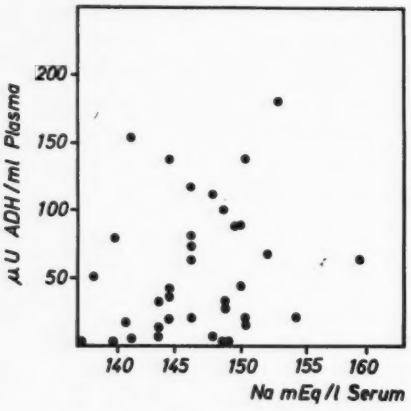

FIG. 3. ADH plasma level and sodium concentration in serum.

and ADH concentration, the conclusion can be drawn, that the serum osmolarity regulates the ADH secretion. This parallels the work of Verney (6), who tells of diuretic inhibition brought about by injection of a hypertonic solution into the carotid artery.

The relation of the ADH plasma level shows an essentially greater variance to the serum sodium concentration than it does to the serum osmolarity (Figs. 3, 4). This can be explained on the basis of the fact, that the serum osmolarity is determined essentially, but not solely, by the sodium ion. In addition, the measurement of osmolarity can be made as exact as 1 mOsm, while sodium is only measured from 2–3 mOsm.

Our earlier published results (7) showed that this relationship is not only valid for healthy individuals, but also, to the same degree, for patients who suffered from an edema of cardiac, hepatic or nephrotic origin. When the ADH plasma concentrations of edematous patients were plotted against their serum osmolarity we were able to construct an exponential curve which fell within the deviation range of the normals. Consequently, ADH is not a primary cause for water retention in patients with edema but the result of the increase in serum osmolarity. The rise of serum osmolarity was the result of sodium retention influenced by the mineralocorticoids of adrenal cortex. This mechanism acts until the serum osmolarity returns to normal following a water retention.

The only exceptions to this rule are seen in acute and long-lasting changes of plasma volume resulting, for example, from a plasma infusion or an abdominal paracentesis with a subsequent increase or decrease in blood volume. Such blood volume changes, according to our previous

studies (7), produce a decrease or increase of ADH content in plasma above or below the ADH value found normally with serum osmolarity. Therefore, it can be concluded that the secretion of ADH is regulated by plasma

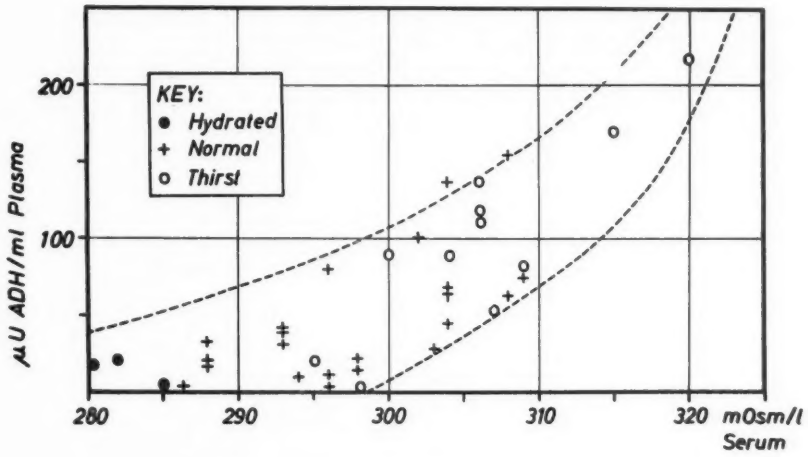

FIG. 4. ADH plasma level and serum osmolarity.

volume as well as by serum osmolarity. This was earlier suspected by Leaf and Mamby (8) and by Gauer and Henry (9).

SUMMARY

With a highly sensitive and specific bioassay on the toad the ADH plasma concentrations in healthy human individuals under various conditions of hydration and dehydration were determined.

A significant regression ($p < 0.001$) from the serum osmolarity to antidiuretic plasma activity, together with a weaker correlation to serum sodium, was found statistically.

This is a direct proof of Verney's assumption, that the ADH secretion from the neurohypophysis is governed by the serum osmolarity. The ADH plasma levels were found to range between <10 and 220 μU/ml.

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EFFECT OF HYPOPHYSECTOMY ON THE INSULIN REQUIREMENT AND RESPONSE TO FASTING OF "TOTALLY" PANCREATECTOMIZED RATS

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THE important role of the anterior pituitary gland in diabetes has been studied in several different species (1, 2, 3, 4). Such investigations in the rat, however, have been few and have employed primarily alloxan-diabetic animals (5, 6, 7).

Recently it was demonstrated that after removal of more than 99.5% of the pancreas the rat develops severe diabetes and dies within 48 hours in coma if not treated with insulin (8). This paper describes some of the effects of hypophysectomy on the diabetes in this rat. In the hypophysectomized-pancreatectomized rats the insulin requirement was reduced, hyperglycemia and glycosuria did not occur during fasting, and there was no ketonuria or excess accumulation of fat in the liver and kidneys when insulin was withheld.

EXPERIMENTAL

Female albino rats, weighing 120 to 150 gm., of the Instituto de Biologia y Medicina Experimental were used in these experiments. The method for removing more than 99.5% of the pancreas and the postoperative care were described earlier (8). The diet (4370) consisted of casein, 20%; L-cystine, 0.3%; dextrin, 66.7%; corn oil, 8%; salts, 4%; and all vitamins needed by the rat (8). When fed to pancreatectomized rats, pancreatin[3] was added to the diet, 10 mg./gm. of dry diet. The operated animals (which were never given food *ad libitum*) were tube-fed an aqueous suspension of the diet (0.5 gm./ml.) twice daily with an interval of 6 or more hours between meals. Insulin was always given 1 to 2 hours before feeding in the morning and less than one-half hour in the afternoon. By the 4th postoperative day all rats received daily 18 units of regular insulin and 7 gm. of food.

Hypophysectomy was performed via the parapharyngeal approach while the rat was anesthetized with sodium pentobarbital and ether. Hypophysectomies were done be-

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[3] 4 U.S.P. Pancreatin, "Viokase" (Viobin Corp., Monticello, Illinois).

tween the 4th and 14th day after pancreatectomy. Crystalline penicillin G, 8,000 units, was injected daily starting 3 days before hypophysectomy. Unless otherwise stated, complete removal of the whole pituitary gland was achieved in all hypophysectomized rats as evidenced at autopsy by the absence of pituitary tissue in or near the sella turcica when examined with a microscope at 10× magnification. Sham-hypophysectomy consisted of making the hole in the skull but not cutting the dura or disturbing the pituitary gland.

On the day of hypophysectomy food and insulin were not given. The next day 2 gm. of food and 1 unit of insulin were given in the morning and 3.5 gm. and 1.5 units in the afternoon. Thereafter the hypophysectomized-pancreatectomized rats were given twice daily 3.5 gm. of food and 1.5 units of regular insulin. After hypophysectomy the food was always given before the insulin in order to prevent the severe hypoglycemic reactions which sometimes occurred if the insulin was injected first.

Laparotomy consisted of anesthetizing the rat with ether, opening the abdomen, exposing the stomach, duodenum, spleen, and transverse colon to air for 20 minutes, and then closing the abdomen with sutures.

The nitrogen content of urine, food, and feces was determined by the microkjeldahl method, glucose in urine by Benedict's quantative method (9), glucose in blood by the method of Haslewood and Strookman (10), and ketone bodies in urine by the qualitative nitroprusside test (Rothera) or the quantitative method of Van Slyke (11).

For histological study, the kidney and liver were fixed in Helly's solution and 10% formalin. Tissues mounted in paraffin were sectioned at 3 micra and stained with hematoxylin and eosin and by the PAS method of Hotchkiss-McManus (12). Frozen sections of tissue fixed in 10% formalin were stained by the oil red-O method of Wilson (13). The degree of the fatty changes in the tissues, based on the severity and extent of the lesions, was measured according to the following scale: 0, none; 1, slight; 2, moderate; 3, intense.

RESULTS

Insulin Requirement: Hypophysectomy reduced the requirement of insulin[4] from 18 to 3 units in pancreatectomized rats tube-fed 7 gm. of food each day (Fig. 1). The reduction in insulin requirement was evident within 24 hours after hypophysectomy; if the insulin dosage was not reduced, the rats had severe hypoglycemic convulsions. The survival after hypophysectomy was greater when the interval between the 2 operations was increased. Five out of 11 rats died when the interval was 4 days, whereas only 1 out of 11 died when it was 14 days. About 50% of the rats had ketonuria on the day of hypophysectomy, on which day they were given no insulin. On the next day and thereafter the doubly operated rats did not have ketonuria. All rats that had pituitary fragments died in diabetic coma.

The daily glycosuria of 6 hypophysectomized-pancreatectomized rats tube-fed 7 gm. of food was 0.10 ± 0.02 gm.[5] when 3 units of insulin were injected daily (5 days) and 0.34 ± 0.11 gm. when 1 unit was injected daily (3 days). Although the glycosuria was only 0.53 ± 0.06 gm. when 0.3 unit was injected daily, 2 rats died on the second day with blood glucose levels in excess of 1,000 mg./100 ml. None of these rats had ketonuria. During the

[4] The amount of insulin needed to maintain glycosuria at less than 0.2 gm./day.

[5] Mean ± standard error.

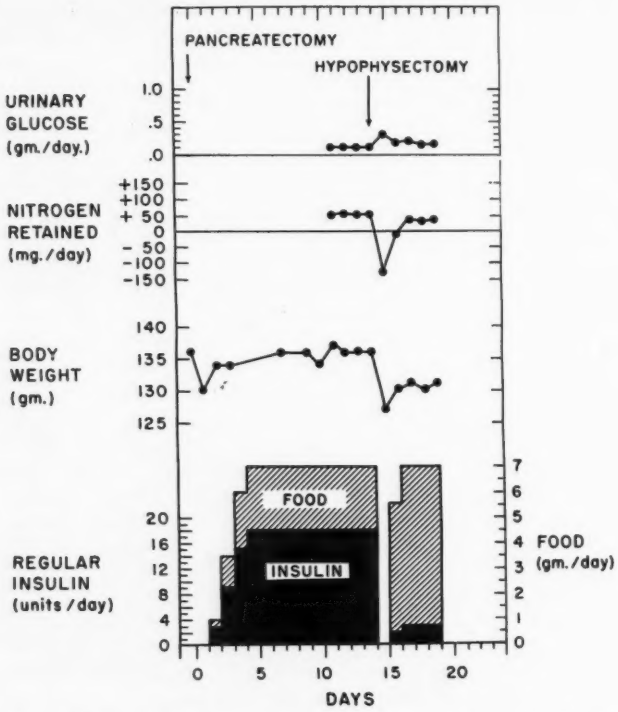

FIG. 1. Effect of hypophysectomy in 10 pancreatectomized rats.

first 3 days after increasing the insulin dosage to 3 units per day the daily glycosuria averaged 0.25 ± 0.03 gm. When pancreatectomized rats with intact pituitary glands were given 4 units of insulin per day, the daily glycosuria averaged 0.4 gm.; 2 units, 1.0 gm.; and 1 unit, 1.4 gm. The daily

TABLE 1. EFFECT OF STOPPING INSULIN TREATMENT IN TUBE-FED PANCREATECTOMIZED AND HYPOPHYSECTOMIZED-PANCREATECTOMIZED RATS[a]

Groups	No. of rats	Average survival, days	Blood glucose mg./100 ml.[b]		Daily urinary excretion of glucose, gm./100 gm. body weight[b]		Ketonuria, No. of rats		Amount of fat[c]	
			Initial[d]	Final	With insulin	Without insulin[e]	With insulin	Without insulin	Kidney	Liver
Pancreatectomized[f]	5	1	279± 41	1,020± 100	0.07± 0.01	1.50± 0.03	0	5[h]	2–3	2–3
	1	8	318	327	.03	2.10	0	0	0	0
Hypophysectomized-pancreatectomized[g]	6	1.5	(200)	1,370± 190	0.03± 0.01	0.83± 0.11	0	0	0–1	0–1

[a] Tube-feeding of 7 gm. of food per day was continued after insulin therapy was stopped.
[b] Mean ± standard error.
[c] Amount of fat, graded from 0 to 3, observed in histological sections stained by Wilson's oil red-O method.
[d] Determined in a.m., 17 hours after last injection of insulin.
[e] Average for survival period.
[f] 95% of the pancreas was removed about 10 weeks before this study; these rats had been given 12 units of regular insulin per day. For 7 days prior to the present study, they also received injections of growth hormone (obtained from C. H. Li), 0.1 mg./day. There was no effect of this or greater amounts (up to 3 mg./day) of growth hormone on the diabetes in the pancreatectomized rats.
[g] More than 99.5% of the pancreas was removed about 3 weeks before and the pituitary about 1 week before this study; these rats received 3 units of regular insulin per day after hypophysectomy.
[h] The urinary excretion of ketone bodies during the last 24 hours averaged 11 mg./100 gm. body weight.

TABLE 2. EFFECT OF HYPOPHYSECTOMY ON RESPONSE TO FASTING IN PANCREATECTOMIZED RATS: BODY WEIGHT, SURVIVAL, BLOOD GLUCOSE, AND FAT IN KIDNEYS AND LIVER

Operation[a]	No. of rats	Body weight, gm.[b]		Body weight lost, % of initial weight (A)		Average survival, days	Blood glucose, mg./100 ml.[c]				Amount of fat in kidneys and liver[f]
		At first operation[c] (A)	At start of fast[d]	Since first operation	During fast		At start of fast[b]	2 days before final	1 day before final	Final[e]	
Pancreatectomy	3	132 ± 6	124	20	14	2	343 ± 9	343 ± 9	331 ± 23	312 ± 10	3
Sham-hypophysectomy	4	117 ± 4	113	33	30	5	336 ± 28	286 ± 80	165 ± 57	31 ± 8	0–2
Sham-hypophysectomy	8	121 ± 7	116	22	17	6	182 ± 47	29 ± 4	20 ± 3	13 ± 1	0–1
Laparotomy	7	118 ± 2	118	25	25	7[g]	54 ± 5	45 ± 4	59 ± 8	47 ± 7	1

[a] Hypophysectomies and sham-hypophysectomies were done on the 5th day after pancreatectomy.
[b] Determined 17 hours after last feeding.
[c] Mean ± standard error.
[d] The fast was begun either on the 8th day after pancreatectomy or on the day of laparotomy.
[e] Determined 4 or less hours before death.
[f] Amount of fat, graded from 0 to 3, seen in frozen sections stained by Wilson's oil red-0-method.
[g] Killed on 7th postoperative day.

glycosuria in these rats was 0.8 gm. during the first 3 days after the insulin dosage was increased to 4 units per day.

Blood Glucose: Hypophysectomy did not have an immediate effect on the blood glucose level. The fasting blood glucose concentration[6] in 14 pancreatectomized rats was 318 ± 14 mg./100 ml.[5] before and 296 ± 24 at 18 hours after hypophysectomy[7]. Four or more days later, when the doubly operated rats were given daily 3 units of insulin and 7 gm. of food, the average fasting blood glucose was lower, 202 ± 24 in 18 rats. Although there were marked differences between rats, as much as 300 mg./100 ml., the variation for each rat was small (20 ± 4% in 12 rats studied on 3 consecutive days). The fasting blood glucose level and insulin requirement were not affected by sham-hypophysectomy.

Withdrawal of Insulin: Hypophysectomy did not prolong the survival or prevent the development of hyperglycemia (1,370 mg./100 ml.) in tube-fed pancreatectomized rats when insulin therapy was stopped (Table 1). The pituitary extirpation did reduce the amount of glucose excreted in the

TABLE 3. EFFECT OF HYPOPHYSECTOMY ON RESPONSE TO FASTING IN PANCREATECTOMIZED RATS: URINARY EXCRETION OF GLUCOSE, NITROGEN, AND KETONE BODIES

Operation[a]	No. of rats	Average survival, days	Urinary excretion, mg./100 gm. body weight[b]					
			Glucose		Nitrogen		Ketone bodies	
			Total	Daily	Total	Daily	Total[c]	Daily
Pancreatectomy								
Sham-hypophysectomy	3	2	330 ± 170	180 ± 24	103 ± 56	59 ± 16	86 ± 29 (3)	54 ± 4
Sham-hypophysectomy	4	5	620 ± 140	132 ± 32	293 ± 43	64 ± 12	199 ± 93 (3)	36 ± 19
Hypophysectomy	8	6	<140[d]	<30[d]	183 ± 28	33 ± 4	5 ± 3 (2)	0.4 ± 0.3
Laparotomy	7	7[e]	<120[d]	<20[d]	241 ± 20	36 ± 4	31 ± 15 (4)	5 ± 2

[a] See Table 2 for details of experiment.
[b] Mean ± standard error.
[c] Figures in parentheses indicates number of rats that had ketonuria.
[d] Using the Benedict quantitative method, the minimal amount of glucose that could be measured was generally 20 mg./day and at times, 30 or 40, depending on the amount of urine available for analysis.
[e] Killed on 7th postoperative day.

urine and prevented ketonuria and accumulation of fat in the kidney and liver.

Fasting: Pancreatectomized rats when fasted and not treated with insulin had hyperglycemia, glycosuria, and ketonuria (Tables 2 and 3). On the second day 3 of the rats died in coma and had large amounts of fat in the kidneys and liver. The other 4 rats survived several days longer and then died in hypoglycemic convulsions; they had less fat in the kidneys and liver. The latter group, which weighed less at the time of pancreatectomy, lost more body weight than did the rats that died in coma, 33% vs. 20%.

Pancreatectomized rats deprived of the pituitary gland survived longer, had lower blood glucose levels, excreted less nitrogen in the urine each day, and had essentially no glycosuria and no ketonuria. These rats had only

[6] Unless stated otherwise, the fasting blood glucose samples were taken in the morning 17–19 hours after the last insulin injection and last meal.

[7] At 40 hours after the last meal and last insulin injection.

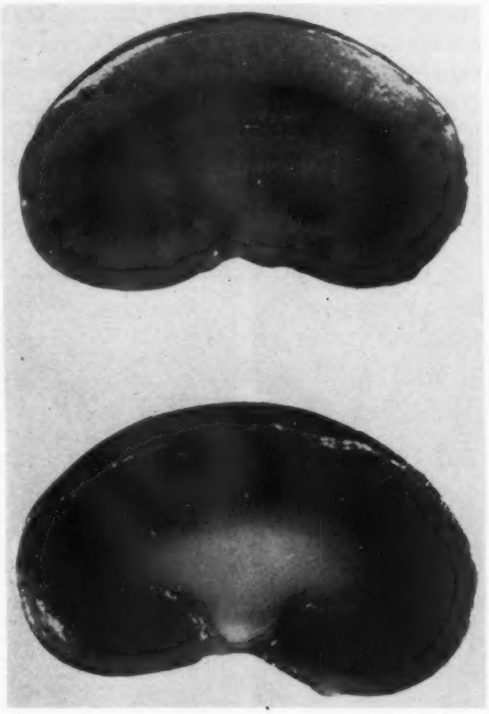

FIG. 2. Longitudinal sections of fresh kidneys of untreated-pancreatectomized (above) and normal (below) rats fasted one day. Note the pale color of the cortex and outer band of the outer medulla of the kidney[8] of the diabetic animal.

FIG. 3. Longitudinal section of kidney of untreated-pancreatectomized rat fasted 2 days. Oil red-0 (Wilson), ×8.

slight traces of fat in the kidneys and livers. Their average blood glucose during the last few days of the fast was less than 30 mg./100 ml.; some rats had levels of less than 20 without spontaneous convulsions.

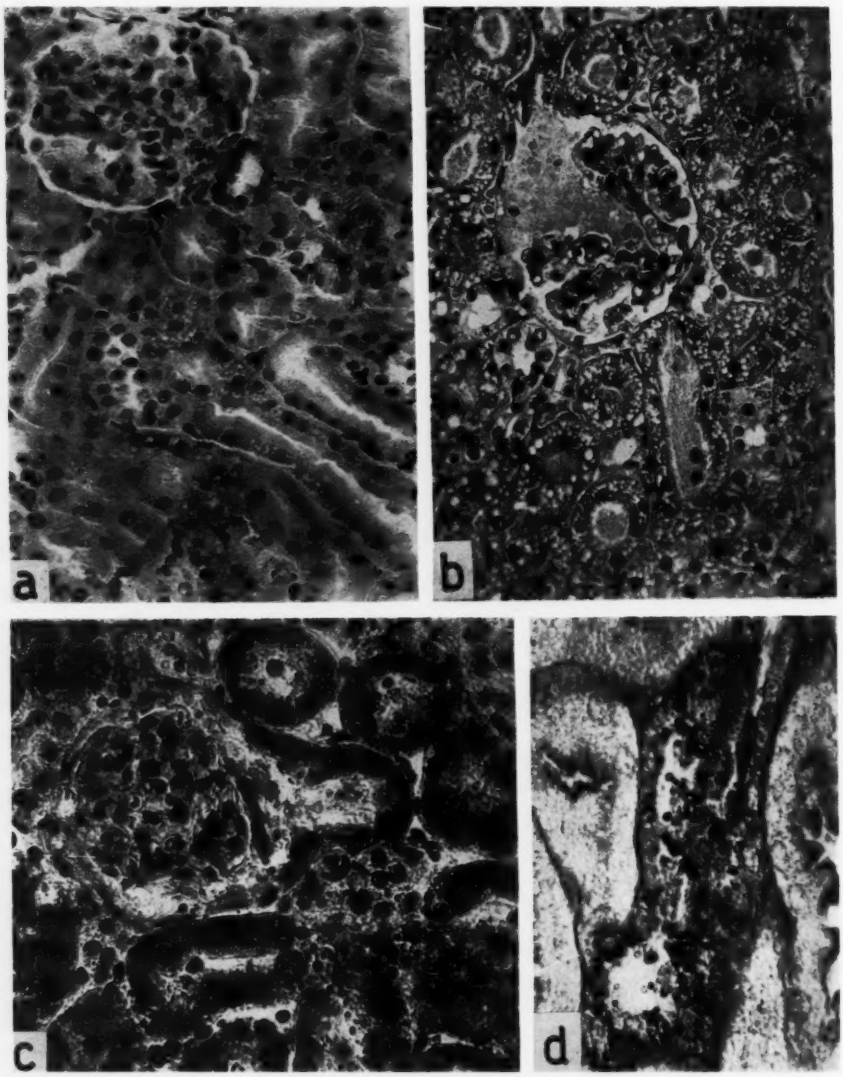

FIG. 4. Kidney. (a) Laparotomized rat fasted 7 days. Hematoxylin and eosin, ×335. (b) Untreated-pancreatectomized rat fasted 2 days. Hematoxylin and eosin, ×335. (c) Fat in renal tubules of untreated-pancreatectomized rat fasted 2 days. Oil red-O (Wilson), ×335. (d) Glycogen in renal tubules of untreated-pancreatectomized rat fasted 2 days. PAS (Hotchkiss-McManus), ×900.

The rats subjected to laparotomy were killed on the seventh postoperative day (Tables 2 and 3). They survived longer and lost less body weight and less nitrogen than did the pancreatectomized rats that died in hypoglycemic convulsions. Their average blood glucose ranged from 45 to 60 mg.%, about twice that in the hypophysectomized-pancreatectomized rats. Half of the laparotomized rats had small amounts of ketone bodies in the urine, whereas all had traces of fat in the kidneys and liver.

Fat in Kidneys and Liver: The kidneys of pancreatectomized rats that died in diabetic coma were very pale. Longitudinal sections of fresh kidney revealed that the cortex and the outer band of the outer medullary zone,[8] both normally brown, were almost white (Fig. 2). Frozen sections stained by Wilson's oil red-O method demonstrated large amounts of fat in the cortex and in the outer band of the outer medullary zone and smaller amounts in the inner band; there was very little fat in the inner medullary zone (Fig. 3). Microscopically the fat was located in the basal and middle portions of the tubular cells (Fig. 4-b,c). Accumulation of fat was greatest in the convoluted tubules and least in the thin segments of the loops of Henle and the collecting tubules. Fat was seldom seen in the glomeruli or in the lumina of the tubules. Sections stained with hematoxylin and eosin demonstrated congestion in the capillary loops of the glomeruli and eosinophilic granular exudative material in Bowman's space compressing the capillaries (Fig. 4-b). Small granules of glycogen, stained by Hotchkiss-McManus PAS method, were seen in the thin portion of Henle's loop and the epithelial cells of the pelvis (Fig. 4-d). The glycogen content was increased in the fatty kidneys.

The livers also were pale in the rats that died in diabetic coma. The hepatic cells of these livers were hypertrophied, globular in shape, and had well defined borders (Fig. 5-b). The cytoplasm contained numerous vacuoles of small and medium size which encircled the nuclei; the latter were centrally located. Occasionally pyknotic cells were seen near the centers of the lobules. The sinusoid capillaries were collapsed, whereas the central and portal veins were dilated. Frozen sections stained with Wilson's oil red-O method demonstrated numerous fat droplets in the hepatic cells (Fig. 5-c). In fatty livers of grade 3 (Tables 1 and 2) the fat-containing cells were located throughout the liver lobules, whereas in livers of grades 1 and 2 the fat was located in the central zones of the lobules. At times a few droplets of fat were seen in the central veins. There was very little, if any, glycogen in the livers.

DISCUSSION

These experiments in the rat confirm many of the observations made in other species concerning the effects of hypophysectomy in pancreatecto-

[8] According to the classification of K. Peter as described by Trueta *et al.* (14)

mized animals: (1) Hypophysectomy reduces but does not abolish the need for insulin when the animals are given carbohydrate (15, 16); (2) hypophysectomy reduces gluconeogenesis in fasting animals deprived of insulin, as

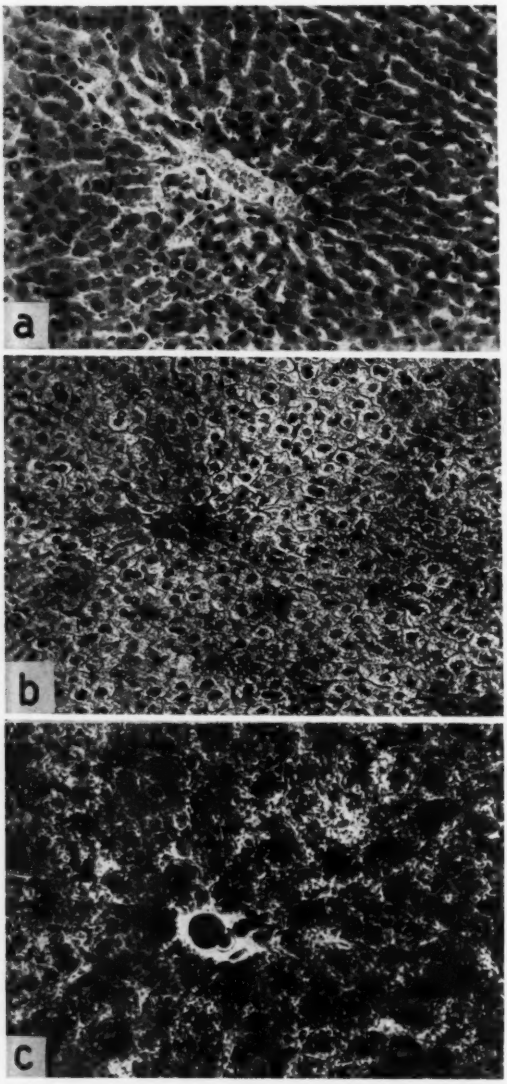

FIG. 5. Liver. (a) Laparotomized rat fasted 7 days. Hematoxylin and eosin, ×235. (b) Untreated-pancreatectomized rat fasted 2 days. Hematoxylin and eosin, ×235. (c) Fat in liver of untreated-pancreatectomized rat fasted 2 days. Oil red-0 (Wilson), ×235.

manifested by the decrease in blood glucose, glycosuria, and urinary nitrogen excretion (2, 3); (3) hypophysectomy prevents the development of ketonuria and fatty livers in animals deprived of insulin, whether fasted or fed (17, 18).

In the present study it was observed that half of the pancreatectomized rats when fasted died on the second day with terminal *hyper*glycemia, whereas the other half died on the fifth day in *hypo*glycemic convulsions; the hypoglycemia developed during the last 2 days of life (Table 2). Except for a pronounced difference in initial body weight, the two groups were indistinguishable during the first one and one-half days of the fast. More recent studies in our pancreatectomized rats have clearly demonstrated that whether diabetic rats will die in coma when deprived of insulin depends largely upon their body fat content; rats with large amounts of body fat die in coma within 2 days when deprived of insulin, whereas those with depleted fat stores do not develop ketosis (ketonuria, hyperketonemia). The differences between the two groups of fasting pancreatectomized rats in the present study are then undoubtedly due to the differences in body weight: The rats that died in coma probably developed more severe ketosis than the others because they had more body fat; their initial body weight was 12% greater. The lighter rats lived until their protein stores were depleted; they died several days later in hypoglycemia, having lost 33% of their body weight. This large body weight loss probably accounted for their surviving a shorter time than did the laparotomized controls (19).

There was an immediate accumulation of fat in the cells of the renal tubules of rats deprived of insulin. These fat-containing cells appeared to be normal except for the presence of fat. Fat was seldom seen in the lumina of the tubules or in the glomeruli and was never found in the urine. The excess fat disappeared when insulin was given and its accumulation was prevented by hypophysectomy.

Since either hypophysectomy or insulin prevents the development of fatty livers and ketonuria, as well as fatty kidneys, the accumulation of fat in the kidney of the uncontrolled diabetic is most likely a manifestation of increased lipid mobilization and metabolism. Generally it is said that ketone body formation occurs primarily if not exclusively in the liver (20, 21). However, it has been demonstrated that the rat kidney, in addition to its ability to utilize fat (22) and ketone bodies (23), can also form ketone bodies (23, 24). In rat kidney slices it was observed that 50% of the acetate metabolized was converted to acetoacetate (23). Whether the kidney in severe diabetes contributes ketone bodies to the blood for use by other tissues is not known.

Increased amounts of intracellular fat in the kidney tubules have also been observed in human diabetics (25), dogs with spontaneous diabetes (26), pancreatectomized dogs (27), and diabetic dogs treated with growth

hormone (28). Small increases of fat in the kidney have been found in normal female guinea pigs (29) and rats (30) injected with anterior pituitary extract.

SUMMARY

Hypophysectomy reduced but did not abolish the need for insulin in "totally" pancreatectomized rats when tube-fed. The reduction in insulin requirement occurred within 24 hours after the removal of the pituitary gland. When insulin was withheld and tube-feeding continued, the doubly operated rats died within 2 days with blood glucose levels of 1,370 mg./100 ml. Hyperglycemia, glycosuria, and increased nitrogen excretion did not occur in the hypophysectomized-pancreatectomized rats when fasted. Hypophysectomy prevented the development of ketonuria, fatty kidneys, and fatty livers in pancreatectomized rats, fasted or fed, when deprived of insulin.

Acknowledgment

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HEPARIN-INDUCED BLOCK OF THE LEUKOCYTE RESPONSE TO CORTISONE[1]

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PREVIOUS reports (1, 2, 3) have indicated that heparin prevents the eosinopenia or the complete leukocyte response to at least some stressor agents. There is not agreement, however, on the mechanism by which heparin blocks the response. Hamilton and Lowenthal (3) showed that pretreatment of rats with 150 I.U. heparin/100 gm. body weight was not effective in preventing the leukocyte response to 12.5 mg. cortisone/100 gm. body weight but did prevent the response to 5 mg./100 gm. This observation suggested a dose-relationship between cortisone and heparin which is the subject of the present report.

METHODS

Male rats weighing between 150–250 gm. were used in the experiments. In most cases they were isolated and kept in a quiet room at 26–28° C for several days before being used. The experiments were carried out at the same time of day to eliminate the effects of diurnal variation on the leukocyte count. Cortisone ("Cortone," Merck and Co.) and heparin (Connaught Laboratories, Toronto) were given subcutaneously into the flank. When both drugs were administered they were given on contralateral sides. Control groups received corresponding volumes of saline as a placebo. In the first experiment the drugs were given at the same time, in the second experiment the cortisone preceded the heparin injection by 30 to 150 minutes. Four hours after the cortisone injection (or in the groups receiving only heparin or saline, 4 hours after their respective injections) the rats were anesthetized by the intra-peritoneal injection of 30 mg. pentobarbital sodium and blood samples were obtained by cardiac puncture. Approximately 1 ml. of blood was collected in a syringe moistened with heparin and transferred to a sampling tube. The total leukocyte count was done in duplicate and the mean value used for the data. The duplicate counts were required to check within 10%. A differential count was performed in which 400 cells were studied, but, for reasons explained below, only the total leukocyte response is presented in this report.

RESULTS

The neutrophil count is normally low in the rat and this was confirmed in the present experiment. The neutrophil level was not altered significantly by either cortisone or heparin in any group studied. Because the eosinophil and monocyte levels were also low, and because the unpaired sample technique was necessary with rats, the changes in these cell types

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were usually not statistically significant. There was, however, a tendency for these cells to be reduced from the control level. The lymphocytes comprise the greatest percentage of leukocytes in the rat and the response to stress is characterized by a significant lymphopenia maximal at about 4 hours. In the present study a significant decrease in the total leukocyte count from the control level at this time was a reliable indication of a stress response.

The control leukocyte count was 17,629 per mm.^{3} (Fig. 1). When heparin

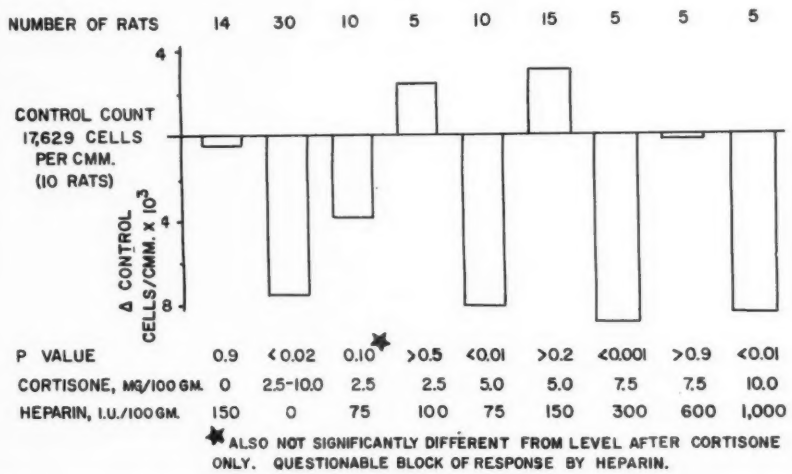
NUMBER OF RATS 14 30 10 5 10 15 5 5 5
4
CONTROL COUNT 17,629 CELLS PER CMM. (10 RATS)
Δ CONTROL CELLS/CMM. x 10^3
4
8
P VALUE 0.9 <0.02 0.10* >0.5 <0.01 >0.2 <0.001 >0.9 <0.01
CORTISONE, MG/100 GM. 0 2.5-10.0 2.5 2.5 5.0 5.0 7.5 7.5 10.0
HEPARIN, I.U./100 GM. 150 0 75 100 75 150 300 600 1,000
* ALSO NOT SIGNIFICANTLY DIFFERENT FROM LEVEL AFTER CORTISONE ONLY. QUESTIONABLE BLOCK OF RESPONSE BY HEPARIN.

FIG. 1. The differences in total leukocyte count between control and animals receiving simultaneous injections of cortisone and/or heparin at varying dose levels.

at a dose level of 150 I.U./100 gm. body weight was given to 14 animals there was no significant difference from control. Cortisone injections in doses ranging from 2.5 to 10 mg./100 gm. body weight produced a marked decrease in the leukocyte count in 30 rats. Because no significant difference was demonstrated in the magnitude of the response to the different doses of cortisone, the results have been grouped together. When 2.5 mg. cortisone/100 gm. was administered with 75 I.U. heparin/100 gm. the leukocyte level was not significantly different from that of either the control group or the group receiving only cortisone. When the dose of heparin was increased to 100 I.U./100 gm., however, there was a definite block of the response to 2.5 mg. cortisone ($p>0.50$). It was necessary to increase the dose of heparin to 150 I.U. to prevent the response to 5 mg. cortisone and to 600 I.U. to prevent the response to 7.5 mg. cortisone. With 10 mg. cortisone/100 gm. no block was demonstrated with heparin doses up to 1,000 I.U./100 gm. It was thus observed (Fig. 1) that, at least up to 7.5 mg. cortisone/100 gm., the leukocyte response to higher doses of the hormone could be prevented by increas-

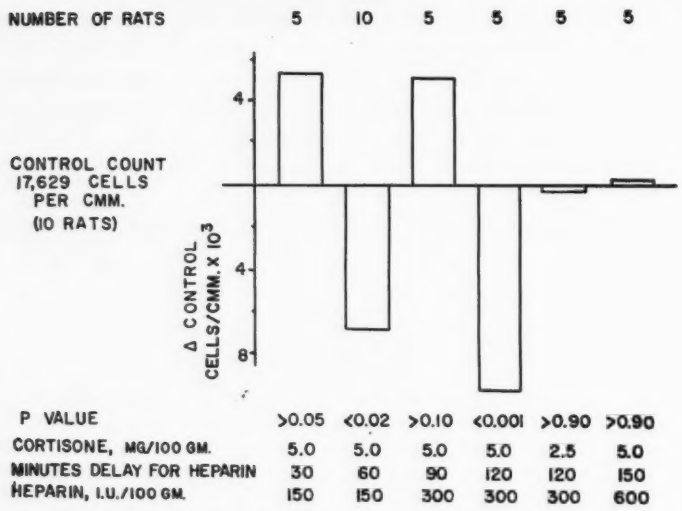

FIG. 2. The differences in total leukocyte count between control and animals receiving heparin and cortisone at different dose levels and varying time relationships.

ing the dose of heparin when the two agents were administered simultaneously.

Figure 2 presents the data obtained from studies designed to investigate the effect of giving the cortisone injections at various times preceding the heparin injection. It was seen that 150 I.U. heparin/100 gm. prevented the response to 5 mg. cortisone/100 gm. when its injection followed the cortisone by 30 minutes but not if the delay was extended to 60 minutes. Heparin at a dose level of 300 I.U./100 gm. was effective against 5 mg. cortisone/100 gm. if the delay was extended to 90 minutes but not when it was 120 minutes. When the dose of cortisone was reduced to 2.5 mg./100 gm., however, 300 I.U. heparin/100 gm. was effective after 120 minutes. It was finally observed that the response to 5 mg. cortisone/100 gm. was prevented by giving 600 I.U. heparin/100 gm. 150 minutes after the cortisone administration. When the period of delay for the heparin injection was prolonged, therefore, it became necessary to increase the dose of heparin to inhibit the leukopenia resulting from a given dose of cortisone in the rat.

DISCUSSION

The effectiveness of heparin in preventing the leukocyte response to cortisone has been shown to be related to the dose of heparin employed against a given dose of cortisone. Heparin can prevent the response even when it is given after the administration of cortisone. No previous reports were found in the literature of the leukocyte response to stress being pre-

vented or abolished by any procedure instituted after the administration or release of adrenocortical steroids. The full significance of this finding is not appreciated at the present time, but it is the basis for the suggestion that the heparin-induced block is at some intermediate stage of action in the target organ.

It was observed that in most groups when heparin prevented the leukocyte response to cortisone there was a tendency for the count to be higher than in the control group. In no instance was the increase statistically significant, but the observation deserves some consideration. It is difficult to handle rats and to make injections without producing at least a mild stress-response. The control leukocyte count, therefore, may have reflected a mild stress in the animals. If this were true, a higher count would be expected when heparin was effective in blocking the stress response, and such a tendency was observed (Figs. 1 and 2). Godlowski (1) presented data which also showed a tendency for the eosinophil count to be slightly higher than control values in men and dogs receiving heparin and stressor agents or heparin alone.

The mechanism of heparin interference with the leukocyte response to stress is not yet clear. Godlowski (1) reported that large doses of heparin alone mobilized eosinophils from the intestinal mucosa and that this action balanced the eosinopenic response to stress. In most cases the increases were slight, however, and as suggested above, they might be the result of blocking the action of endogenous adrenocorticoids. Samter (4) showed an insignificant rise in the eosinophil level after heparin administration in guinea pigs. In the experiments with rats reported here and with preliminary experiments on rabbits (unpublished results) heparin in doses sufficient to block the leukocyte response to stress did not produce a significant leukocytosis, eosinophilia or lymphocytosis.

Godlowski (5) assigned part of the action of heparin to inhibiting proteolytic enzymes which actively destroy the circulating eosinophils. No discussion of the mechanism for the effect of heparin on the lymphopenia following stress was offered by Godlowski.

Weissbecker and Schroter (2) believed that heparin interfered with the stimulation of the adrenal cortex by ACTH. However, Fekete (6) and Morris, Lowenthal and Hamilton (7) reported that heparin did not prevent adrenocortical stimulation, as measured by adrenal ascorbic acid depletion, in response to endogenous or exogenous ACTH. The demonstration that heparin prevented the response to cortisone indicated further that ACTH inactivation was not the mechanism of heparin interference with the stress response.

An earlier suggestion (3) that heparin might interfere with cortisone transport in the blood was not supported by the present work. It was demonstrated that the leukopenic response to stress could be prevented by giv-

ing the heparin up to 2½ hours after cortisone. Most of the cortisone was probably absorbed and transported to the target organs by that time.

Essellier, *et al.* (8), believed that the eosinopenic response to cortisone was caused by increased reticuloendothelial activity since blocking the RES prevented the response. It has been shown that the RES granulopexic activity can be blocked by heparin (9). An antagonism of the actions of growth hormone (STH) and cortisone on the eosinophil count has been reported (10). It is of interest that the effect of trypan blue (8) or STH (10) could be broken through by administering larger doses of cortisone in the same way that the effect of heparin could be overcome by increasing the dose of cortisone. These similarities support the hypothesis that heparin may act on the target organ in preventing the leukocyte response to cortisone.

Eyring and Dougherty (11) suggested that histamine may be involved in the peripheral stress response and Swingle *et al.* (12) have demonstrated that histamine is able to produce an eosinopenia in adrenalectomized dogs. The formation of a complex between heparin and amine-containing basic materials has been demonstrated (13, 14, 15). If histamine is involved in the leukopenic response to stress the formation of such a complex might block the leukocyte response to cortisone. However, the possibility that heparin interfered with another intermediate step in the peripheral response to stress can not be disregarded at the present time.

SUMMARY

Experiments are described which showed that heparin administration could prevent the leukocyte response to cortisone in rats. With increased doses of cortisone, at least up to 7.5 mg./100 gm. body weight, an increased dose of heparin was required to block the response. If the heparin administration followed the cortisone injection, an increased dose of heparin was required to prevent the response to a given dose of cortisone as the period of delay was increased. The response to cortisone was prevented by giving relatively large doses of heparin up to 2½ hours after the cortisone administration. It was concluded that the site of action is at the target-organ, through interference with some intermediate step in the sequence of events initiated by cortisone administration.

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SURFACE-BINDING OF THYROXINE BY THYROIDAL PROTEINS[1]

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DURING normal hormonal biosynthesis, thyroxine is apparently formed within the matrix of the large thyroglobulin molecule (1). It has been generally believed that thyroxine is thus sequestered within the follicle until enzymatic hydrolysis of thyroglobulin leaves the small molecular hormone free to diffuse into the circulation (2, 3). However, the findings of Leblond and Gross (4), and of Tong, Taurog and Chaikoff (5) suggest that thyroxine liberated by the hydrolysis of thyroglobulin need not diffuse freely from the follicle. These authors demonstrated, in the thyroid glands of rats, thyroxine (and diiodotyrosine) which was readily extractable from unhydrolyzed glandular extracts with butyl alcohol. This "free" thyroxine, though comprising only a small proportion of total glandular iodine, was present in concentrations many times greater than the concentration of butanol-extractable thyroxine in the plasma. It was suggested that glandular "free" thyroxine might in fact be loosely bound to thyroidal protein in much the way that thyroxine is bound to the proteins in plasma (5).

There has recently been found in plasma a protein moiety to which, at physiological concentrations, the majority of the plasma's thyroxine is bound (6, 7, 8). In plasma, thyroxine partitions between this thyroxine-binding protein (TBP) and other proteins (principally albumin) according to their concentrations, their avidities for thyroxine, and the concentration of thyroxine itself (9).

It seemed possible, therefore, that there might occur within the thyroid gland a protein or proteins similar to TBP, at least with regard to capacity for binding thyroxine. Such proteins, if present in adequate quantity, could compete for thyroxine with the proteins of plasma, and could thereby serve to maintain thyroid:plasma concentration gradients for "free" thyroxine. The findings herein described indicate that purified thyroglobulin has

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considerable ability to bind thyroxine in a loose, butanol-dissociable link, and, in this sense, can effectively compete for the hormone with several of the proteins in plasma.

METHODS

Proteins were extracted from human and from sheep thyroid glands. Human preparations were derived from pooled specimens of gland obtained from several patients during excision of toxic or benign nodular goiters. Following removal of specimens for pathological examination, tissues were stored at −30° F until extraction of proteins was carried out. Fresh sheep thyroid glands were obtained from animals killed by exsanguination at the abattoir, and were transported to the laboratory on cracked ice prior to storage in the deep freeze.

Extracts of human thyroid gland were prepared by homogenizing the tissues in veronal buffer, pH 8.6, and in acetate buffer, pH 4.5 (ionic strength 0.10). Homogenates were adjusted to a final thyroid:medium ratio of 10 parts per 100. Following centrifugation, the still-cloudy supernatants were employed in studying thyroxine binding, as described below.

Purified thyroglobulin was prepared from three batches of human and from two batches of sheep thyroid glands, according to the methods described by Derrien, Michel and Roche (10). Salting-out was effected with either ammonium sulfate or potassium phosphate. Following several re-precipitations and re-solutions, the isolated protein was dialyzed against isotonic sodium chloride in a cold chamber. In early experiments, the dialyzed protein was lyophilized for storage. However, in confirmation of earlier reports, dried thyroglobulin did not retain its solubility in physiological media (11). Subsequent preparations were, therefore, kept in solution, frozen at −30° F.

Purified porcine thyroglobulin was obtained from a commercial source.[2]

The ability of thyroidal extracts and solutions of thyroglobulin to bind thyroxine, in comparison to the binding abilities of several of the plasma's proteins, was assessed according to principles described in detail by Freinkel, Dowling and Ingbar (9). In brief, this consisted of assessing the quantitative distribution of added I^{131}-labelled thyroxine following the resolution of protein mixtures by electrophoresis on filter paper (Whatman #3). Veronal buffer, pH 8.6, ionic strength 0.05, and acetate buffer, pH 4.5, ionic strength 0.10, were employed. Radioactivity on electrophoretograms was determined by means of an automatically-recording strip scanner, and by radioautography. Proteins were localized by staining electrophoretic strips with brom-phenol-blue (12).

The binding of thyroxine to thyroidal proteins during paper electrophoresis was assessed in three systems. In the first, thyroidal extracts or solutions of thryoglobulin (1.0 to 2.0 gm. %)[3] were enriched with I^{131}-labelled thyroxine[4] and sufficient stable thyroxine to achieve concentrations of thyroxine varying from 10 to 80 μg. %. In the second system, thyroidal extracts or solutions of thyroglobulin, comparably enriched with thyroxine, were further supplemented with mercaptalbumin[5] (14) or Cohn Fraction

[2] Kindly supplied by Dr. Robert L. Kroc, Warner-Chilcott Laboratories, Morris Plain, New Jersey.

[3] Protein concentrations were estimated by a modified biuret technique (13).

[4] Chromatographically pure. Obtained from Abbott Laboratories, Oak Ridge, Tennessee.

[5] Kindly provided by Dr. Margaret J. Hunter of the Department of Biophysical Chemistry of Harvard Medical School. Mercaptalbumin was chosen as a standard binding competitor since it had previously been shown to be entirely free of TBP and to retain considerable thyroxine-binding potency (9).

II-III (15), prepared from human plasma. The mercaptalbumin or Fraction II-III were brought up to a concentration of 3.0 gm. % . In these two systems, homologous serum was labelled with radioactive thyroxine and concurrently electrophoresed to serve as a reference marker.

In the third system, the capacity of human and sheep thyroglobulins to displace thyroxine from serum albumin was assessed. Samples of homologous serum were enriched with radioactive and stable thyroxine, and diluted by the addition of equal volumes of either 0.9% NaCl or solutions of thyroglobulin. Following dilution, the distribution of thyroxine was assessed by electrophoresis as described above.

Further experiments were performed to determine the characteristics of the association between thyroxine and thyroglobulin. Solutions of human thyroglobulin (2.0 gm. %)[3] previously shown to bind thyroxine, were enriched with radioactive and stable hormone. Solutions of plasma Fraction IV-6 of Cohn *et al.*, a fraction which has been shown to be rich in the plasma's TBP (9), were similarly supplemented with labelled and unlabelled thyroxine. Aliquots of both solutions were subjected to precipitation and washing with 10% trichloracetic acid. The radioactivity of precipitates and supernatant solutions was assayed in a well-type scintillation counter. Other aliquots of these solutions were extracted with acid-butanol (16), and the counts in the butanol-soluble and insoluble phases were measured. In each instance, the recovered radioactivity was related to the radioactivity of the aliquots prior to precipitation or to butanol extraction. Sufficient counts were observed to reduce the probable counting error to less than 1%.

RESULTS

In terms of electrophoretic mobility and thyroxine-binding characteristics, there appeared to be no significant difference between proteins extracted from human thyroid glands at pH 8.6 and those extracted at pH 4.5. During electrophoresis at pH 8.6, the predominant protein component of both extracts migrated in the zone intermediate between the α_1 and α_2 globulins of the concomitantly electrophoresed sera (Fig. 1). A small portion of the extracted proteins migrated in the albumin zone, and a pale band in the β-globulin was seen to bear the coloration of hemoglobin prior to staining with bromphenol blue. Added I^{131}-labelled thyroxine migrated in association with the principal interalpha protein, and thus displayed an electrophoretic mobility similar to that of thyroxine bound to serum TBP. However, the radioactive band in thyroidal extracts was considerably broader than the TBP band in serum.

Added mercaptalbumin abstracted a small portion of radiothyroxine from the interalpha onto the albumin zone. This was evident on radioactive scanning as a shoulder on the inter-alpha peak.

During electrophoresis at pH 4.5, the principal protein component of thyroidal extracts remained virtually immobile (Fig. 2). In addition, small papilliform anodal projections were seen. Added radioactive thyroxine bore the same configuration as the protein bands. Added mercaptalbumin displaced a portion of the radioactivity to the cathodally-directed albumin zone. Significant quantities of protein or of radioactivity were not seen in the distinctly anodal position occupied by TBP in concomitantly electrophoresed sera.

Thyroxine-binding moieties similar to the TBP of human serum were

found in sera obtained from sheep (not illustrated) and hogs (Fig. 3). In sera from all three species, the major thyroxine-binding protein displayed an electrophoretic mobility, at pH 8.6, in the inter-alpha zone. In the serum of sheep, however, distinct protein bands were not obtained during

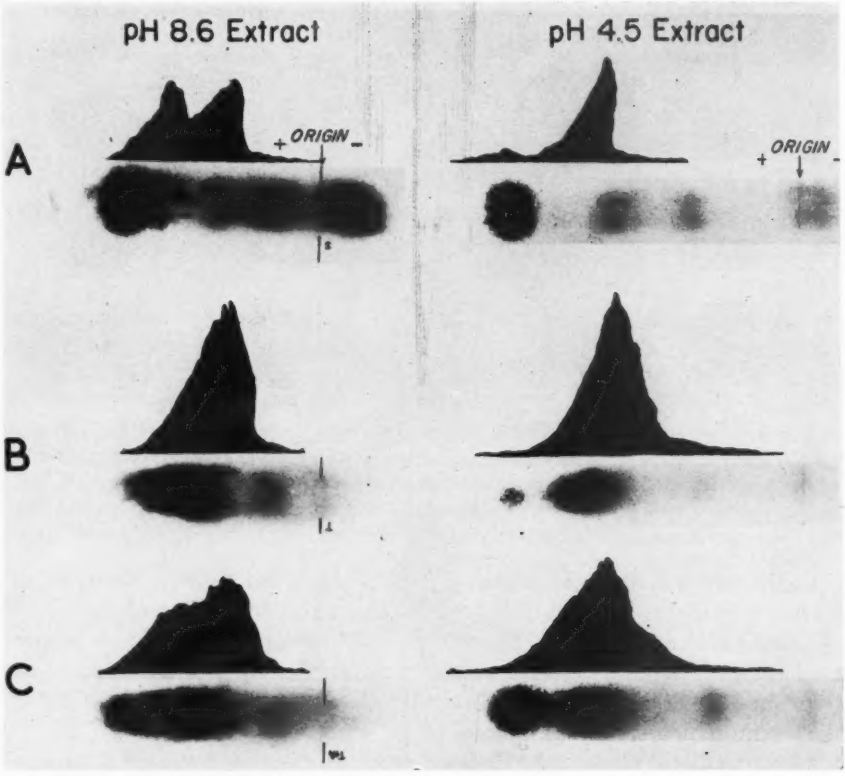

FIG. 1. Electrophoresis at pH 8.6 of human thyroidal extracts prepared in buffers of pH 8.6 and pH 4.5. Electrophoretic distribution of added radio-thyroxine. A, human serum; B, thyroidal extract; C, thyroidal extract containing added human mercaptalbumin (2.0 gm. %). In each category, upper figure depicts scan of distribution of added radio-thyroxine, and lower figure depicts bom-phenol-blue stain of electrophoretic strip.

electrophoresis in veronal buffer, pH 8.6. In the three species studied, purified thyroglobulin displayed the same mobility at pH 8.6 as homologous TBP (Figs. 3 and 4).

Added radioactive thyroxine was bound to purified thyroglobulin during electrophoresis, and, as in thyroidal extracts, the radioactive band was somewhat broader than that associated with TBP in serum. Upon addition of human mercaptalbumin, a portion of the radioactive thyroxine was displaced from the thyroglobulin onto the albumin zone. The magnitude of the

displaced portion varied from species to species and from batch to batch within any species. Complete abstraction of radiothyroxine from thyroglobulin by albumin was not seen. In general, thyroglobulin prepared with potassium phosphate competed with albumin more effectively than thyro-

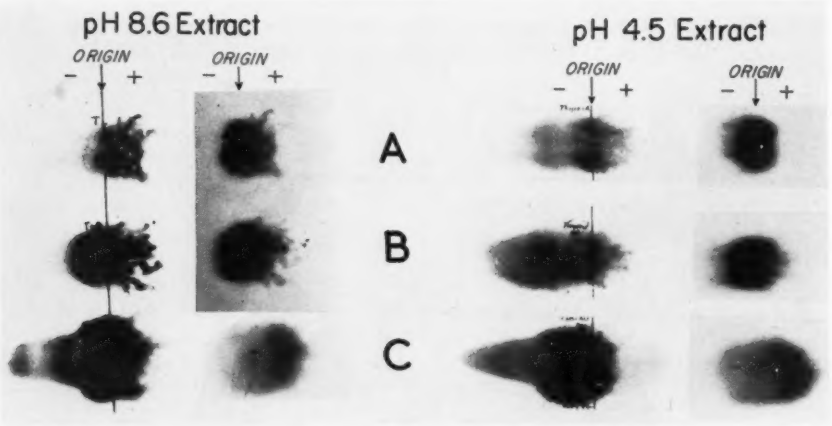

FIG 2. Electrophoresis at pH 4.5 of human thyroidal extracts prepared in buffers of pH 8.6 and pH 4.5. Electrophoretic distribution of added radio-thyroxine. A, thyroidal extract; B, thyroidal extract containing added human mercaptalbumin (2.0 gm. %); C, human serum. In each category, figures on the left and right depict brom-phenol-blue stain and radioautograph, respectively, of electrophoretic strip.

globulin which had been prepared with ammonium sulfate. Addition of human γ-globulins did not displace radiothyroxine from thyroglobulin.

During electrophoresis at pH 4.5, purified thyroglobulin and its associated radiothyroxine remained virtually immobile. No protein or radioactive component displayed the distinctly anodal migration of the TBP in the serum which served as control (Figs. 4 and 5).

In contradistinction to the findings in serum diluted with saline, human and sheep sera diluted with solutions of homologous thyroglobulin displayed diminished binding of radiothyroxine in the albumin zone and increased binding in the inter-alpha zone.

The thyroxine which had been added to solutions of thyroglobulin was completely precipitable with trichloroacetic acid, and bound thyroxine could be entirely extracted with acid butanol. Thyroxine added to solutions of Cohn Fraction IV-6 displayed similar characteristics.

DISCUSSION

As judged by electrophoretic criteria, proteins contained in extracts of human thyroid gland are capable of binding added radioactive thyroxine. At pH 8.6, these proteins migrate principally in the zone between the α_1 and α_2 globulins of concomitantly electrophoresed serum. Since this coin-

cides with the electrophoretic mobility of the thyroxine-binding protein of plasma (TBP), it seemed possible that binding of thyroxine by thyroidal extracts might result from inclusion of TBP within the extracts. This could have resulted from blood trapped within the gland during surgical removal,

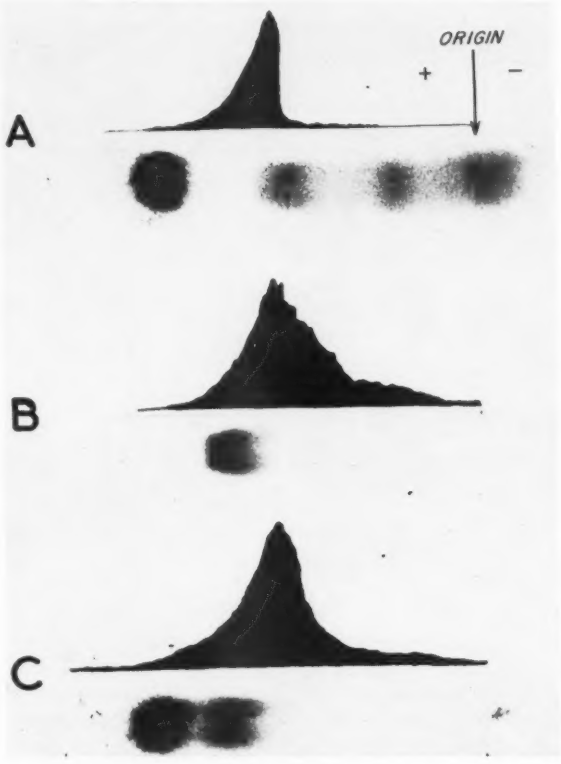

FIG. 3. Electrophoresis at pH 8.6 of purified hog thyroglobulin. Electrophoretic distribution of added radio-thyroxine. A, hog serum; B, hog thyroglobulin (1.5 gm. %); C, hog thyroglobulin (1.5 gm. %) containing added human mercaptalbumin (3.0 gm. per cent). In each category, upper and lower figures depict radioactive scan and bromphenol-blue stain, respectively, of electrophoretic strip.

as evidenced by the presence of small quantities of albumin and hemoglobin in electrophoretograms. Alternatively, as suggested by the data of Boas and Foley (17), TBP might be contained within the thyroidal parenchyma *per se*. Electrophoretic migration at pH 4.5 (18), served to exclude these possibilities. Although anodally-migrating moieties similar to those noted by Boas and Foley were seen, the principal protein and thyroxine-binding components remained virtually immobile, and all components could be readily distinguished from the anodally-migrating TBP of serum.

Attention was next directed toward the possibility that thyroglobulin

itself constituted the thyroxine-binding moiety of thyroidal extracts. Therefore, purified preparations of human, sheep, and porcine thyroglobulin were studied. In all three species, thyroglobulin was found to bind thyroxine and to compete effectively for the hormone with preparations of

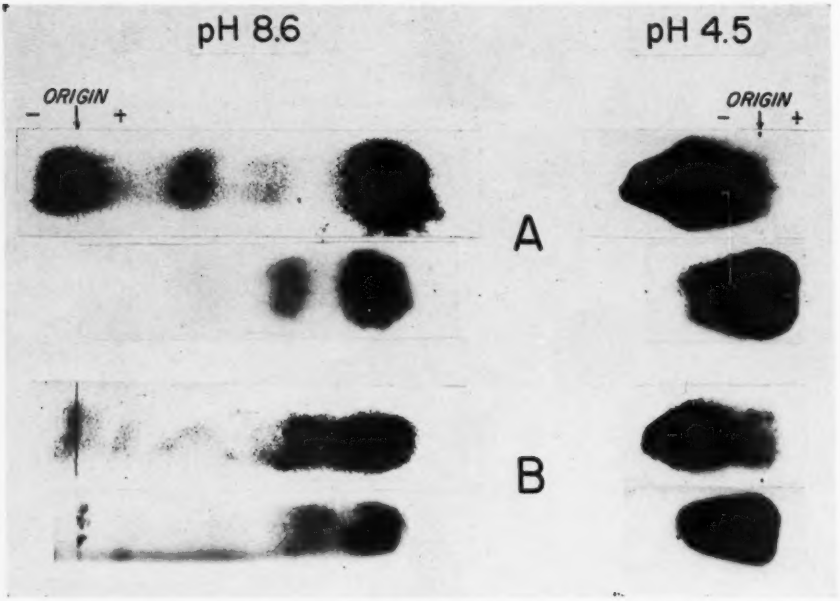

FIG. 4. Electrophoresis of purified human thyroglobulin at pH 8.6 and pH 4.5. Electrophoretic distribution of added radio-thyroxine. A, human serum; B, human thyroglobulin (1.5 gm. %) containing added human mercaptalbumin (3.0 gm. %). In each category, upper and lower figures depict brom-phenol-blue stain and radioautograph, respectively, of electrophoretic strip.

human albumin and gamma globulin. In addition, when added to serum, human and sheep thyroglobulins were capable of displacing thyroxine from albumin.

Once again it seemed possible that preparations of thyroglobulin were contaminated with TBP, since the salting-out curves of the two proteins partially overlap (19). This possibility could not be excluded by electrophoresis at pH 8.6, since in all three species, the TBP and thyroglobulin migrated similarly at this pH. However, significant quantities of TBP could not be demonstrated during electrophoresis at pH 4.5. At this pH, no component of the specimens of thyroglobulin exhibited the distinct anodal migration displayed by TBP in concomitantly electrophoresed homologous sera.

Finally, the ease of removal of bound thyroxine by albumin and by acid-butanol indicated that binding of thyroxine by thyroglobulin is a surface phenomenon and does not result from exchange of either labelled thyroxine or its radioactive iodine for their pre-existing stable counterparts within the matrix of the thyroglobulin molecule.

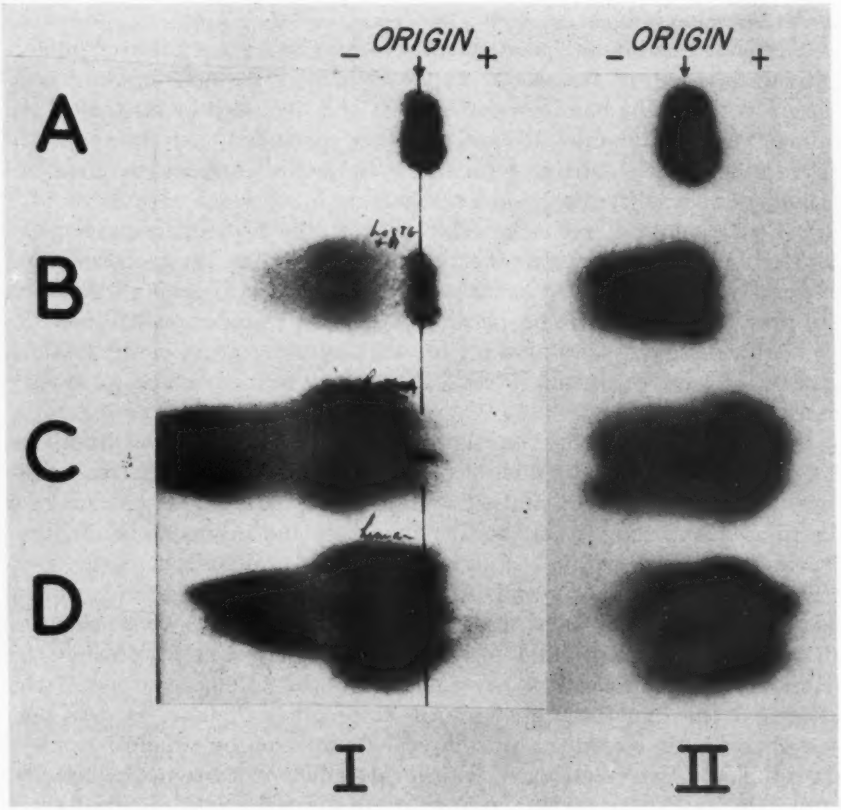

FIG. 5. Electrophoresis at pH 4.5 of purified hog thyroglobulin. Electrophoretic distribution of added radiothyroxine. A, hog thyroglobulin (1.5 gm. %); B, hog thyroglobulin (1.5 gm. %) containing added human mercaptalbumin (3.0 gm. %); C, hog serum; D, human serum. I. Brom-phenol-blue stain. II. Radioautograph of electrophoretic strip.

Thyroglobulin thus satisfies previously defined criteria for an active thyroxine-binding material (9), and appears to be the principal thyroxine-binding moiety of thyroidal extracts. The present findings further indicate that proteins which are electrophoretically similar to human TBP occur in the serum of sheep and hogs, and that in each instance, the homologous

thyroglobulin exhibits similar electrophoretic mobility at pH 8.6. In this regard, it is noteworthy that thyroglobulin of the rat has been found to migrate at pH 8.6 just behind albumin (20). This is the zone in which the the thyroxine-binding moieties of rat's serum are localized (21).[6] The significance of this sharing by thyroglobulin and TBP of both electrophoretic migration at pH 8.6 and capacity to bind thyroxine is unknown, but seems of considerable interest.

The thyroxine-binding potency of thyroglobulin has not yet been quantitatively assessed. Nevertheless, it appears to be of the same order of magnitude as that of human mercaptalbumin, and to exceed by far that of the human gamma globulins. It can be further concluded that the avidity of thyroglobulin for thyroxine is far less than that of comparable quantities of human plasma fractions, such as Fraction IV-6, which are rich in TBP (9). Nevertheless, it seems possible that at the high concentrations of thyroglobulin which exist in the thyroid gland (17), thyroglobulin could effectively compete for thyroxine with the small quantities of TBP which are present in the perfusing plasma. A binding equilibrium between the two proteins might thus account for the thyroid:plasma concentrations gradients for loosely-bound ("free") thyroxine which have been previously described (4, 5).

In view of the foregoing considerations, it seems possible that thyroxine liberated during the hydrolysis of thyroglobulin is not entirely free to diffuse into the circulation. Rather, such intrathyroidal thyroxine may be distributed according to the relative quantities and thyroxine-binding potencies of the TBP within the perfusate and the proteins, principally thyroglobulin, within the thyroid. Alteration in the relative abundances or relative binding affinities of these components might then be expected to alter either the static thyroid:plasma ratio of loosely-bound thyroxine, the fractional rate of release of thyroxine, or both. Attempts to assess the validity of this hypothesis are currently in progress. Preliminary data suggest that despite continued hypophyseal suppression by triiodothyronine, the administration of estrogen, with its attendant increase in the activity of plasma TBP (22), effects an augmentation of the rate of turnover of intrathyroidal I^{131} (23).

Finally, recent reports have indicated that the glandular content of iodine exerts a regulatory influence on the further accumulation of iodine which is independent of hypophyseal control. Several authors have suggested that the regulatory agents within the gland might be those iodinated compounds which are hormonally active in the periphery (24, 25). If this be true, it would seem probable (by analogy with their peripheral effectiveness) that loosely-bound hormone within the gland would be more active

[6] Thyroxine-binding moieties have been investigated in the sera of a variety of additional species. Data obtained will be the subject of a subsequent report.

than hormone bound within the thyroglobulin molecule. Thus alterations in hormone-binding equilibria between thyroidal and plasma proteins, by inducing changes in the glandular content of loosely-bound hormone, might influence not only the rate of turnover, but also the rate of accumulation of glandular iodine.

Clearly, the foregoing considerations concerning the physiological consequences of thyroxine-binding by thyroglobulin are speculative. Nevertheless, they afford a conceptual basis for attempts to elucidate further the origin and significance of glandular "free" thyroxine.

SUMMARY

Thyroidsl proteins are capable of binding thyroxine in a loose, butanol-dissociable link. This capacity appears to reside in the thyroglobulin molecule itself.

Thyroxine-binding by thyroidal extracts and by solutions of purified thyroglobulin apparently does not result from the inclusion of proteins identical to the plasma's thyroxine-binding protein (TBP).

The avidity of thyroglobulin for thyroxine is less than that of serum TBP, exceeds that of the gamma globulins, and is about the same as that of human albumin.

The possible role of thyroxine-binding equilibria between thyroidal and plasma proteins in maintaining thyroidal:plasma concentration gradients for "free" thyroxine and in regulating the glandular metabolism of iodine has been discussed.

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EFFECTS OF RENIN AND THYROXINE IN RATS TREATED WITH CORTICOSTEROIDS AND IN RATS WITH REGENERATING ADRENALS[1]

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ADMINISTRATION of renin to rats pretreated with salt and desoxycorticosterone (DCA), cortisone or 17-hydroxycorticosterone (cortisol) (1, 2, 3) elicits an eclampsia-like state associated with widespread acute destructive vascular lesions. This condition is attributable, in part, to the enhanced pressor effect of renin associated with the presence of excess adrenosteroids (4). Recently, a somewhat similar syndrome has been described in rats given thyroxin together with salt and DCA plus cortisone (5). In this situation the pressor and nephrosclerotic actions of thyroxine (6) may be additive to those of the steroids or it may act by specific sensitization of pressor mechanisms. The present experiments constitute a comparative study of the effects of thyroxine and renin in animals treated with adrenosteroids and also in animals with regenerating adrenals; the latter condition is stimulated by thyroxine (7) and is believed to be associated with hyperfunction or imbalance in steroid secretions (8).

GENERAL METHODS

All the rats (Sprague-Dawley strain) were uninephrectomized on the right side, given 1% saline as drinking fluid, fed Purina Fox Chow and housed in an air-conditioned room. In experiments on rats with adrenal enucleation, the operation was performed according to the method of Ingle and Higgins (9) on the left side with simultaneous right adrenalectomy. The steroids (desoxycorticosterone acetate, cortisol acetate) were administered subcutaneously as saline suspension; the daily dose of each steroid was 1 mg. per day in 0.1 cc of fluid. The thyroxine solution was prepared as follows: thyroxine was dissolved in water by addition of 0.1N NaOH, then dilute HCl was carefully added until persistence of a slight opalescence; the final solution was made up with saline so that 0.1 cc contained 100 μg. of thyroxine. Partially purified renin (step 6) was prepared according to the method of Haas, Lamfrom and Goldblatt (10); the solution contained 75 Goldblatt dog units per cc. In view of the susceptibility of the thyroxine treated rats to hyperthermia, blood pressure (11) was measured only in a few instances. In some experiments, rats were placed in metabolism cages for determination of urine output.

At autopsy selected tissues were removed, fixed in Susa's fluid and weighed; sections were prepared and stained with periodic acid fuchsin (PAS). On histologic examination of the kidney, the degree of nephrosclerosis was graded from 0 to 3, taking as criteria fibrinoid necrosis, casts, tubular dilatation and cellular accumulation.

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EXPERIMENTAL AND RESULTS

Comparative Effects of Renin and Thyroxine in Steroid Treated Rats

The acute and chronic effects of thyroxine and renin were compared in 2 series of experiments under conditions similar to those used previously (1, 5). In the first series, renin and thyroxine were given over short periods of time. 24 female rats weighing between 105 and 130 gm. were divided evenly according to weight into 3 groups treated as follows: group I, DCA+cortisol+thyroxine; group II, DCA+cortisol+renin; and group III, DCA +cortisol. Renin or thyroxine were given on the 10th through the 16th day of steroid treatment. In the second series, all treatments were started simultaneously. 22 rats ranging from 88 to 114 gm. in body weight were divided into 2 groups of 11 each. The animals of group I received DCA +cortisol+thyroxine; the initial dose of thyroxine of 250 μg. was increased to 500 μg. on the 6th day. The animals of group II received DCA+cortisol +renin; renin was given first in a daily dose of 28 units which was increased to 42 units on the 9th day and to 70 units on the 12th day. The increase in dosage of renin had the purpose of overcoming the effects of any antirenin formed. The experiment was terminated on the 16th day.

In series I, the first detectable effect was on body weight (Table 1). While it remained constant in the control group, it was markedly increased in the renin group; this effect was already maximal within the first 8 hours of renin treatment when the mean body weight increased from 118 gm. to 140 gm. and remained at this level until death. This sudden gain, present in most of the rats, was the result of an increase in water intake and a decrease in the flow of urine, which took a dark reddish appearance. Fluid retention in the form of anasarca was present in 90% of the renin-treated rats and was associated with a mortality of about 50%. In comparison with this group, the thyroxine-treated rats lost weight and showed less mortality and fluid retention; fluid retention which was restricted to the pleural and/or peritoneal spaces was not sufficient to counter the wasting

TABLE 1. COMPARATIVE EFFECTS OF RENIN AND THYROXINE IN STEROID-TREATED RATS

Group and treatment	Body weight*		Mortality in %	Fluid retention in %	Nephrosclerosis†	Heart	Kidney
	A	P				Gm. % of body weight	
			1st series				
I. Steroids plus Thyroxine	127±6.0‡	113±5.3	20	20	2.3	.47±.01	1.09±.06
II. Steroids plus Renin	118±3.2	140±7.2	50	90	2.7	.35±.01	.87±.03
III. Steroids	119±4.7	118±4.2	0	0	1.7	.35±.01	.83±.02
			2nd series				
I. Steroids plus Thyroxine	101±2.8	100±5.9	40	60	1.8	.50±.02	1.31±.04
II. Steroids plus Renin	101±2.9	101±5.5	50	90	1.7	.35±.01	1.07±.07

* Body weight Ante (A) and Post (P) renin or thyroxine treatment in experimental group or at similar intervals in control group. In the second series, (A) and (P) correspond to initial and final body weight.
† Average values of lesions graded from 0 to 3+.
‡ Mean ± standard error.

effect of thyroxine. In Table 1 heart and kidney weights are expressed in percentage of body weight. Final body weight is not representative because of water retention in some of the rats, so that in series I we used in these calculations the body weights at the beginning of renin or thyroxine treatment. Results expressed this way or in terms of absolute weights demonstrate the cardiotrophic and nephrotrophic effects of thyroxine; renin treatment did not alter significantly heart and kidney weights.

On macroscopic examination, only thyroxine- and renin-treated groups had detectable lesions. Kidneys had an irregular surface mottled with hemorrhages which were petechial in the thyroxine rats and extensive, sometimes involving the whole kidney, in the renin rats; hemorrhages in heart, intestine and brain predominated also in the renin group. Thymus was completely atrophic and lesions of mesenteric arteries were absent probably as a result of cortisol treatment (12). On histologic examination all the rats, including the controls had nephrosclerosis; as seen in Table 1 it was equally severe in the renin and thyroxine groups. In both groups there were arteriolar necrosis, casts, tubular dilatation or atrophy, and interstitial cellular accumulation, in the same degree of severity. The glomerular lesions were however qualitatively different. In the renin group, the glomerular tuft was infiltrated with PAS-positive material inside (so-called "colloid thrombi") and around the capillaries; this was superimposed on the lesions produced by the steroid treatment. On the other hand, thyroxine caused merely an intensification of the glomerular lesions seen in DCA-produced nephrosclerosis with increased thickening of the capsular membrane, hypertrophy of the tuft cells with focal fibrinoid necrosis and accumulation of fibrinoid in the capsular space.

The results obtained in the second experimental series in which thyroxine and renin were given simultaneously with the steroids from the beginning of the experiment showed only small, quantitative differences from those obtained above (Table 1). Although initial and final body weights were the same, there was an initial loss of weight in both groups, presumably due to the catabolic effect of the steroids and of thyroxine or renin; on about the 8th day, fluid retention started to develop and contributed to a progressive return of the body weight to its original level. Since the toxicity of renin can be demonstrated only after steroid sensitization, the effects of this hormone became evident as early as the 8th day of treatment when a rat died with all the signs of the DCA-renin disease. Although the incidence of mortality and fluid retention in the thyroxine treated group was higher than in the previous series, the morphological changes were similar to those described above.

Effects of Thyroxine in Rats with Adrenal Enucleation

In a preliminary experiment adrenal enucleation was performed in 16 rats weighing 140–164 gm. evenly divided into 2 groups. Group I was kept

as control while Group II was treated with thyroxine in a daily dose of 100 μg. Thyroxine had no appreciable effects on body growth but definitely accelerated and intensified the hypertension (Table 2). Adrenal enucleation alone did not elicit any marked increase in blood pressure, presumably because the rats were near the adult stage (13). Starting on the 20th day, mortality was high in the thyroxine group as a result partly of hyper-

TABLE 2. EFFECTS OF THYROXINE IN RATS WITH ADRENAL ENUCLEATION

Group and treatment	Blood pressure on day			Heart	Kidney	Nephrosclerosis severity†
	7th	14th	20th	gm. in % body weight		
I. Adrenal enucleation	151±7.7*	166±6.4	169±8.1	.30±.009	.63±.02	.4
II. Adrenal enucleation plus Thyroxin	181±7.1	216±5.6	245±3.3	.40±.03	1.08±.18	2.5

* Mean + standard error.
† Mean of lesions graded from 0 to 3+.

thermia during blood pressure determinations and partly of the severity of the hypertensive disease; no rat died in Group I. The experiment was terminated on the 30th day when only 3 rats were alive in Group II. At autopsy fluid retention was present only in Group II; heart and kidney weights were larger and nephrosclerosis more severe in Group II than in Group I, which accords with blood pressure values. Adrenal weight was slightly greater in Group II (average 39 mg.) than in Group I (average 31 mg.); inversely thymus was smaller in Group II (average 210 mg.) than in Group 1 (average 341 mg.). From this, it appears that the enucleated adrenal is capable of secreting cortisol-like steroids in response to thyroxine treatment. It should be noted that in spite of the marked hypertension no periarteritis nodosa-like lesions were present, suggesting also secretion of cortisol-like compounds which are known to inhibit those lesions. The vascular lesions seen were those of acute arteriolar fibrinoid necrosis as noted in DCA-renin disease.

From this experiment it was not clear whether thyroxine had any sensitizing effect on the secretions of the enucleated adrenal or stimulated the regenerating adrenal to produce more steroids or possessed a direct hypertensive and nephrosclerotic effect. The following experiment was therefore devised. Forty rats weighing 40 to 60 gm. were divided into 5 groups which were operated and treated as indicated in Table 3. Thyroxine was started at the dose of 12.5 μg. which was increased successively to 50 μg. on the 10th day and to 100 μg. on the 20th day. The experiment was terminated on the 33rd day when the rats in Group II (adrenal enucleation+thyroxine) started to die. Blood pressure was not measured.

From the initial and final body weight values (Table 3) it can be seen that adrenal enucleation alone had no inhibitory effect on growth but that thyroxine produced some inhibition, particularly when associated with

adrenal enucleation. Individual growth curves in the thyroxine treated rats showed an irregular pattern; after reaching a plateau around the 20th day, body weight was subject to fluctuations associated with retention of fluid followed by a sudden loss in weight and disappearance of excess body fluid. An example is provided by a rat in Group II: its initial weight of 53 gm. increased gradually during the first 16 days up to 111 gm. where it re-

TABLE 3. COMPARATIVE EFFECTS OF THYROXINE IN NORMAL RATS AND IN RATS WITH ADRENAL ENUCLEATION OR WITH UNIADRENALECTOMY

Group and treatment	Body weight		Heart	Kidney	Nephrosclerosis severity†
	Initial	Final	Gm. in % body weight		
I. Adrenal enucleation	48±1.1*	152±2.7	.41±.02	1.01±.05	1
II. Adrenal enucleation plus Thyroxine	47± .13	122±8.7	.49±.02	1.39±.04	1.7
III. Uniadrenalectomy	50±2.3	153±5.9	.34±.01	.92±.02	0
IV. Uniadrenalectomy plus Thyroxine	47±1.14	139±5.7	.48±.004	1.4 ±.09	2.4
V. Thyroxine	51±1.9	150±4.3	.51±.01	1.35±.04	2.3

* Mean ± standard error.
† Mean of lesions graded from 0 to 3+.

mained relatively constant during a week, then it suddenly went up to 153 gm. and came back to a value of 113 gm. Such changes while being more marked in Group II, were also present in the other groups receiving thyroxine. At the end of the experiment 50% of the rats in Group II had died. In an unreported experiment, identical to the present one, all rats with adrenal enucleation which received 12.5 μg. of thyroxine died within a week while the other thyroxine-treated rats remained alive. This would suggest a state of adrenal hypofunction—at least regarding cortisol-like steroids—immediately following enucleation, which may be of significance in the pathogenesis of the subsequent hypertension.

At autopsy water retention was seen only in the rats receiving thyroxine; it consisted of ascites, pleural fluid and sometimes subcutaneous edema. It was not unusual to find between 30 and 40 cc. of free fluid. Hemorrhages were present in the heart and intestine but not in the kidneys as is frequently seen in the DCA-renin disease. Thymus was practically nonexistent in the thyroxine treated groups. From Table 3 it can be seen that kidney and heart weights were similarly increased in Groups II, IV and V which all received thyroxine. Nephrosclerosis was absent in Group III, slight in Group I, moderate in Group II, and equally severe in Groups IV and V (Fig. 2). The difference in the degree of nephrosclerosis between Group II and Groups IV and V was noted by 2 different observers. This suggests that the nephrosclerotic effects of thyroxine are at least partly mediated through the adrenals: nephrosclerosis is less severe in rats with adrenal enucleation possibly because the adrenal fragment is already hy-

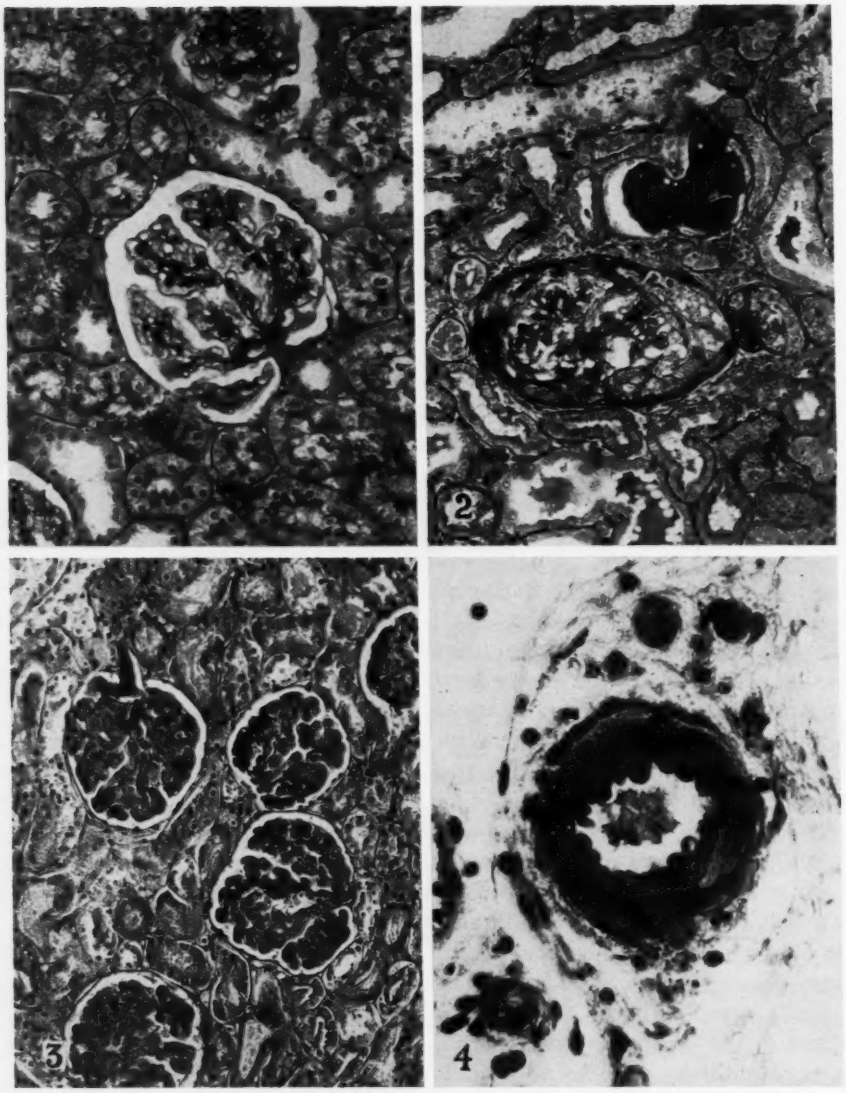

FIG. 1. Kidney of an uninephrectomized untreated rat. (×175)

FIG. 2. Kidney of an uninephrectomized rat treated with thyroxine. Note glomerulus with atrophic tuft, capsular space filled with hyalin material and thickened capsular membrane; also hyalin and protein casts in dilated tubules. (×185)

FIG. 3. Kidney of an uninephrectomized adrenal enucleated rat treated with renin which died during the first 24 hrs of treatment. The glomerular tufts are heavily infiltrated with PAS-positive material. Note a large thrombus in afferent arteriole. The tubular changes are postmortem. (×135)

FIG. 4. Necrosis in mesenteric arteriole. Same rat as in Fig. 3. (×625)

TABLE 4. EFFECTS OF RENIN IN RATS WITH ADRENAL ENUCLEATION

Group and treatment	Body weight		Blood pressure on the 6th week	Urine flow ml. per day		Nephrosclerosis	
	A*	P*		A*	P*	Incidence in %	Severity‡
I. Adrenal enucleation	182±2.6†	192±4.3	191±11.1	40±7.2	38±5.9	60	.59
II. Adrenal enucleation plus Renin	178±9.3	182±9	195±7.4	39±7.3	182±25.5	100	2.3

* A and P as in Table 1.
† Mean ± standard error.
‡ Mean of lesions graded from 0 to 3+.

perfunctional, than in rats with one or both normal adrenals which can be stimulated to hypersecrete. On microscopic examination the lesions produced by thyroxine did not differ qualitatively from those seen in experimental hypertension produced by adrenal enucleation, so that the only difference in results was fluid retention.

Effect of Renin in Rats with Adrenal Enucleation

Adrenal enucleation was performed in 24 rats weighing 60 to 80 gm., which were kept in metabolism cages without treatment until the 6th week when most of the animals had developed hypertension (8). They were then evenly matched according to blood pressure into 2 groups of 11 animals each: animals of Group I were kept as untreated controls while those of Group II received renin subcutaneously for 3 days in 3 daily divided doses totalling 28 units. The survivors were killed on the 4th day and autopsied. Results are summarized in Table 4.

The toxic effects of renin were shown by the death during the first day of 2 rats and by an increased incidence and severity of nephrosclerosis; no definite fluid retention occurred. Daily urine volume increased from 43 ml. to 183 ml. on the 4th day; during this period there was a slight and similar increase in body weight in both experimental and control rats. The 2 rats which died during the 1st day had a small flow (10 and 20 ml. respectively) of dark red urine. Nervous symptoms were observed in 3 rats which at autopsy showed cerebral hemorrhages.

Microscopic examination revealed that the kidneys were the organs most severely affected. These were completely hemorrhagic in the 2 rats which died during the first 24 hours; in others, hemorrhages were primarily petechial. The lesions present in all the rats treated with renin were those of nephrosclerosis associated with occlusion of the tuft capillaries with PAS-positive material (Fig. 3), as in the DCA-renin disease. Small arteries and arterioles in the gastrointestinal tract, mesentery, heart and brain showed lesions of either periarteritis nodosa or acute fibrinoid necrosis (Fig. 4); the first type of lesion, also present in the controls was consequent on adrenal enucleation, while the second type was attributable to renin treatment.

DISCUSSION

The present experiments again demonstrate the eclampsia-like syndrome associated with acute and diffuse vascular lesions caused by subcutaneous injections of renin in corticosteroid-pretreated rats (1–3). This was obtained with a renin preparation other than the one used previously, indicating as did experiments with angiotonin (14) that the syndrome is precipitated in fact by renin. The syndrome occurred also when increasing doses of renin were given chronically together with the steroids. Our previous failure (1), under apparently similar conditions was probably due to neutralization by circulating antirenin of insufficient amounts of renin. In accord with former observations the onset of the syndrome is about the 10th day, i.e., when steroid sensitization has presumably taken place.

Rats made hypertensive by adrenal enucleation also develop the syndrome when given renin over brief periods; since it cannot be elicited by renin alone in uninephrectomized rats on a salt diet (15), it is evident that adrenal regeneration, like exogenous steroids, sensitizes to renin. This sensitization may be due to hypersecretion of corticosteroids or perhaps to an initial adrenal hypofunction. Assuming that the latter is the case, the situation would be equivalent to the well known hypersensitivity of Addisonian patients to renal and vascular actions of DCA. It should be pointed out that the severity of the syndrome obtained is not the same when renin is given after steroid treatment (DCA+cortisol) as after adrenal enucleation. Differences had also been observed when renin was administered to rats pretreated with DCA, cortisone or cortisol (1–3), the milder form being seen in those pretreated with cortisol (3). In all these experimental situations we found the same type of glomerular lesions with PAS-positive material inside and outside the capillaries.

We also confirm that thyroxine given acutely or chronically together with DCA+cortisol elicits a state which resembles the corticosteroid-renin syndrome (5). This condition is characterized by nephrosclerosis, vascular lesions, hemorrhages and fluid retention. However certain dissimilarities distinguish this from the corticosteroid-renin disease. In the latter, the symptoms occur rapidly, fluid retention is generalized (anasarca), the kidneys are frequently hemorrhagic and the glomeruli are infiltrated with PAS-positive material. On the other hand, in the steroid-thyroxine disease, symptoms are delayed, fluid retention is often local, the kidneys are not hemorrhagic and the glomeruli are not thrombosed. These differences may be quantitative only and attributable to the comparatively prompt pressor effect of subcutaneously injected renin, whereas the actions of thyroxine are known to be delayed. These differences however are probably qualitative. Although renin and thyroxine have some pharmacologic properties in common (diuretic, catabolic, increase in blood pressure), these are due to different basic mechanisms and furthermore the two hormones are entirely different in their abilities to elicit hypertension and nephrosclerosis. Renin

injected alone produces only a transient elevation in blood pressure (4); however, in rats made hypertensive by adrenal steroids or adrenal enucleation, or, in completely nephrectomized dogs (16) it elicits respectively the eclampsia-like syndrome or malignant hypertension. In contrast, thyroxine alone, like adrenal steroids, can cause hypertension and nephrosclerosis in uninephrectomized salt fed rats. According to our experience as well as that of others (6, 17), this effect is quite inconstant; it seems that the effective dose is within a relatively narrow range; small doses are ineffective while large doses may not be hypertensive because of a predominant catabolic activity.

The pathogenesis of thyroxine induced hypertension and nephrosclerosis is not clear. It may depend in part upon sensitization to endogenous epinephrine and norepinephrine (18). Morphological studies indicate that thyroxine stimulates the adrenal cortex (19), which may explain why, like DCA induced hypertension, it is dependent on uninephrectomy and salt. Finally it may be related to the nephrotrophic effect of thyroxine; according to Braun-Menendez (20) hypertension would result from a disequilibrium between increased production of an hypothetical "renotrophin" and inability of the kidney to respond to this stimulus. The present experiments do not distinguish between these mechanisms and it may be that all have a pathogenetic role.

SUMMARY

Renin provokes in rats with regenerating adrenals a syndrome of acute renal and vascular damage which although somewhat attenuated resembles the eclampsia-like syndrome elicited by renin in rats pretreated with exogenous corticosteroids. Contrary to previous observation, renin given from the onset of steroid treatment in increasing dosage caused the syndrome after about 10 days, indicating that this period is required for sensitization.

The fact that thyroxine enhances hypertension and vascular damage due to adrenal steroids is confirmed. However contrary to renin, thyroxine does not require sensitization, since alone it produces nephrosclerosis, hypertension and fluid retention in uninephrectomized rats fed one per cent saline. The syndrome provoked by thyroxine (alone, or, combined with steroids or adrenal enucleation) has some similarity (fluid retention and nervous symptoms) to that caused by DCA+renin; it differs in that the renal glomerular lesions are those of nephrosclerosis rather than those of thrombosis and that anasarca is infrequent. It seems likely that these differences are qualitative and due to different basic mechanisms.

Acknowledgment

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EFFECT OF INSULIN ON GROWTH IN FORCE-FED HYPOPHYSECTOMIZED RATS

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SALTER and Best (1, 2) observed a marked increase in the body weight of hypophysectomized rats injected with insulin. The body weight gain, which was one-third fat, was always associated with a great increase in food intake. A similar increase in body weight has been found in hypophysectomized rats tube-fed excess amounts of food (3, 4). In both cases the gain in the nitrogen content of the carcass was very small.

This paper presents the results of a study of the effect of exogenous insulin on the body weight and composition of hypophysectomized rats tube-fed large amounts of food; no differences were observed between the insulin-treated and the untreated rats. The composition of the weight gain in these animals has been compared with that in hypophysectomized rats fed less food but treated with growth hormone.

METHODS

Male rats weighing 120 to 150 gm. of the Instituto de Biologia y Medicina Experimental were used. Hypophysectomy was performed via the parapharyngeal route while the rat was anesthetized with sodium pentobarbital and ether. Complete removal of the whole pituitary gland, as evidenced at autopsy by the absence of pituitary tissue in or near the sella turcica (examined with 10× magnification), was achieved in all operated rats described in this paper. The operated rats were injected daily with 8,000 units of crystalline penicillin G subcutaneously.

The common diet of the institute was fed until one to two weeks before hypophysectomy, and thereafter, diet 4370 (5). The latter is a purified diet consisting of casein, 20%; *l*-cystine, 0.3%; dextrin, 66.7%; corn oil, 8%; salt, 4.0%; and all vitamins needed by the rat in adequate amounts. An aqueous suspension of this diet, 0.5 gm./ml., was tube-fed twice daily with an interval of six or more hours between meals as described earlier (5). Tube-feeding was begun 24 hours after hypophysectomy, with 5 gm. of food given the first day, 6 gm. the next two days, and 7 or more gm. daily thereafter. The operated animals were never given food ad libitum except during the first 24 hours after

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hypophysectomy. They were allowed to drink water ad libitum. The rats were always weighed before the morning feeding.

Regular insulin[3] was injected subcutaneously immediately after each meal. The growth hormone,[4] which was highly purified, was dissolved in slightly alkaline 0.85% NaCl solution and injected intraperitoneally twice daily.

For the nitrogen balance studies each rat was kept in a separate cage suspended over a glass funnel with a wire gauze (1/8″) disc to separate the feces from the urine. The feces were collected daily for seven days; they were then treated with hot 50% H_2SO_4, diluted, and an aliquot taken for nitrogen analysis. The urine was collected daily under a layer of toluene and stored at 5° C; at the end of each collection period, 2–4 days, the urine was diluted and an aliquot was taken for analysis. A 2 ml. aliquot of the diet suspension was collected at each meal; at the end of each collection period, 2–4 days, the pooled samples were treated with hot 50% HS_2O_4, diluted, and an aliquot taken for analysis.

On the day after the last injection and feeding each rat was exsanguinated through a large cardiac incision while under chloroform anesthesia and subjected to immediate dissection and weighing of tissues. All tissues were saved with the carcass for chemical analyses; the blood and the contents of the gastrointestinal tract were discarded. The amounts of nitrogen and fat in the carcass were determined by the method of Kochakian and Stettner (6) and the nitrogen in the food, urine, and stools by the microKjeldahl method.

RESULTS

Experiment A: The body weights of hypophysectomized rats weighing 125–130 gm. were unchanged when 7 gm. of food were tube-fed daily (Figs. 1 and 2). There was an immediate and continuous body weight gain when the food intake was increased to 9 or more gm. (Fig. 1). The body weight gain (Table 1) was associated with significant ($P < 0.05$) increments in the weights of liver, kidneys, gastrointestinal tract, heart, and thigh muscle (Tables 1 and 2). There was no change in the body length (Table 1) or in the weights of the hind leg bones, hind foot, thymus, and seminal vesicles (Table 2). Although the nitrogen content of the animals was significantly increased by force-feeding, the actual increment was very small (Table 3). Only 8% of the body weight gain was protein whereas 66% was fat.

There was no effect of the injected insulin on the body and organ weights and on the body composition of force-fed hypophysectomized rats (Tables 1–3 and Fig. 1).

The nitrogen balance, based on the difference between intake and excretion of nitrogen, was determined in some of the force-fed rats during the course of this experiment. As seen in Table 4, nitrogen was retained throughout the experiment by both insulin-treated and untreated rats. Increasing the food intake above 9 gm./day had only a small effect on the amount of nitrogen retained. Insulin treatment had no significant effect on the retention of nitrogen.

[3] Regular insulin was kindly supplied by Laboratorios Armour of Buenos Aires, Argentina.

[4] Obtained from Dr. C. H. Li of the University of California, Berkeley, California.

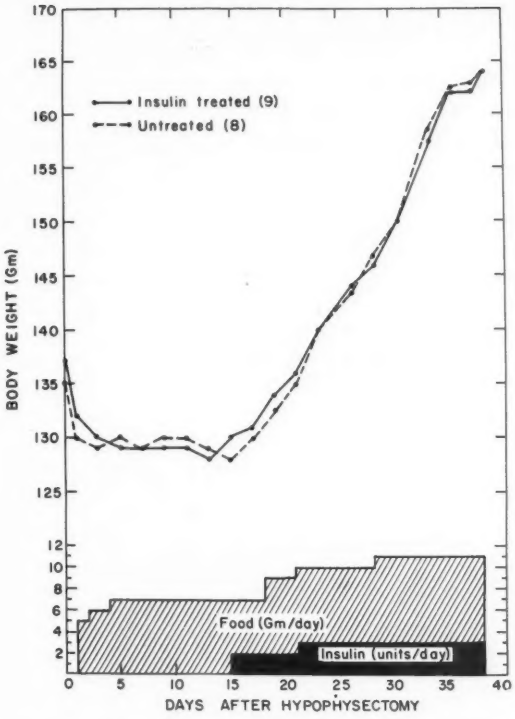

FIG. 1. Experiment A: Effect of Insulin on the body weight gain of tube-fed hypophysectomized rats.

Experiment B: All rats in this experiment were tube-fed 7 gm. of food each day. On this restricted intake, untreated and insulin-treated (2 units/day for 5 days) hypophysectomized rats gained no weight whereas those treated with growth hormone gained 1.1 gm./day (Table 1 and Fig. 2). The daily body weight gain of normal rats tube-fed 7 gm./day was only 0.6 gm.

Growth hormone administration produced a significant gain[5] in the weights of the liver, kidneys, gastrointestinal tract, thigh muscle, hind leg bones, hind feet, and thymus (Tables 1 and 2). There was no weight change in the heart or seminal vesicles.

Although the body weight gain of the growth hormone-treated rats was less than that of the rats fed extra amounts of food (Experiment A), the protein gain was 60% greater (Table 3). There was no increase in the fat content of the growth hormone-treated rats whereas in hypophysectomized rats fed extra food two-thirds of the weight gain was fat.

[5] Since Experiments A and B were run concurrently, the "initial" group for Experiment A serves also as the "initial" group for Experiment B.

DISCUSSION

Salter *et al.* (1, 2) observed that hypophysectomized rats which were injected with insulin ate more food, gained weight, and retained a small amount of nitrogen. In the present study hypophysectomized rats gained weight when tube-fed extra amounts of food, confirming the findings of

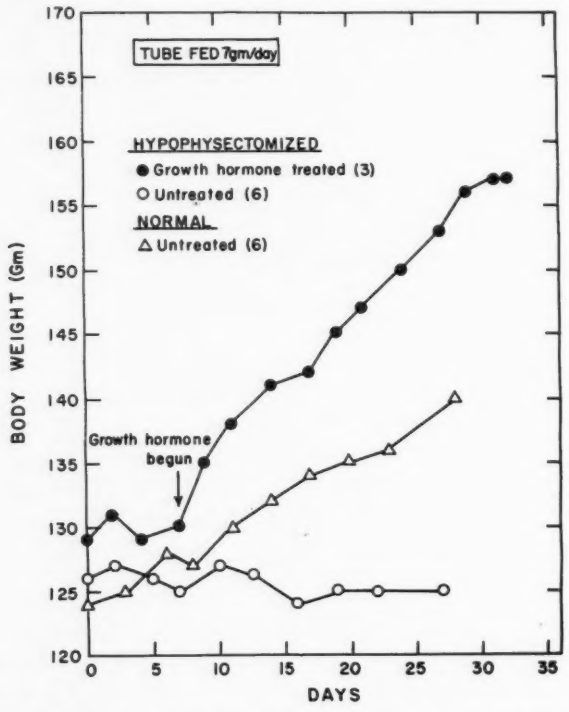

FIG. 2. Experiment B: Effect of growth hormone on the body weight of hypophysectomized rats. Hypophysectomies were done 2 weeks before the start of this experiment. All rats were tube fed 7 gm. of food/day.

others (3, 4). Although the major part of the gain was fat, there was a small increase in the nitrogen content of the animals. The weight gain, the food intake, and the composition of the gain in the overfed hypophysectomized rats were similar to those in the insulin-treated rats of Salter and Best (1). Insulin given in the same dosage as that used by Salter had no effect on the body weight gain or on the retention of nitrogen in the tube-fed hypophysectomized rats.[6] The present findings would suggest that exogenous insulin

[6] It is of interest that the dosage of insulin used by Salter was nearly the same amount as that, 3 units/day, needed for the control of glycosuria (less than 0.1 gm./day) in hypophysectomized-pancreatectomized rats tube-fed 7 gm. of food/day (7). Under these

TABLE 1. EFFECTS OF FORCE-FEEDING, INSULIN, AND GROWTH HORMONE ON BODY AND ORGAN WEIGHTS IN HYPOPHYSECTOMIZED RATS[a]

Groups	No. of rats	No. of days	Average daily food intake, gm./ 100 gm. body weight	Body weight, gm.		Final body length, cm.[c]	Liver, gm.	Kidneys, gm.	Gastrointestinal tract (empty), gm.[d]
				Final	Gain[b]				
EXPERIMENT A[e]									
Hypophysectomized									
Initial	7	0	5.5	128	—	18.3±0.1	4.28±0.21	0.96±0.03	5.65±0.32
Force-fed, untreated	8	22	>6.9	164	32±2	18.6±0.1	6.39±0.73	1.12±0.05	6.74±0.27
Force-fed, insulin	9	22	>6.9	164	30±3	18.3±0.2	6.29±0.66	1.12±0.03	6.76±0.28
EXPERIMENT B[f]									
Hypophysectomized									
Untreated	6	20	5.7	125	−1±2	—	4.72±0.13	0.86±0.02	—
Growth hormone	3	25	4.9	157	27±3	18.8±0.3	6.18±0.29	1.10±0.29	6.87±0.22
Normal									
Untreated	6	28	5.3	140	16±3	—	5.22±0.20	1.17±0.04	—

[a] Mean ± standard error.
[b] Less the gain in weight of the gastrointestinal contents.
[c] Distance between the nose and anus when the anesthetized rat was lying on its back.
[d] The gastrointestinal contents weighed around 4.5 gm. in rats fed 11 gm./day and less than 3 in those fed 7 gm./day.
[e] *Experiment A:* All rats had been hypophysectomized for 15 days; during that time they were tube-fed 7 gm. of food/day (see Fig. 1). At the beginning of the experiment the "initial" group was autopsied. The other two groups were tube-fed 7 gm./day for 3 days, 9 gm. for the next 3, 10 gm. for the next 7, and 11 gm. for the last 9 days. The insulin-treated group was injected with 2 units of regular insulin/day during the first 6 days and 3 units during the last 16 days.
[f] *Experiment B:* The operated rats were hypophysectomized 14 days before the experiment was started. All were tube-fed 7 gm. of food/day before and during the experiment. Growth hormone, 0.1 mg./day, was injected twice daily.

TABLE 2. EFFECTS OF FORCE-FEEDING, INSULIN, AND GROWTH HORMONE ON OTHER ORGAN WEIGHTS IN HYPOPHYSECTOMIZED RATS[a]

Groups[b]	No. of rats	Heart, mg.	Thigh muscle, gm.[c]	Hind leg bones, gm.[d]	Hind foot, gm.	Thymus, mg.	Testes, gm.	Seminal vesicles, empty, mg.
EXPERIMENT A								
Hypophysectomized								
Initial	7	462±13	4.19±0.06	1.09±0.02	1.05±0.02	245±15	1.01±0.18	42±6
Force-fed, untreated	8	552±12	4.97±0.16	1.11±0.03	1.11±0.01	275±27	0.35±0.06	30±2
Force-fed, insulin	9	511± 9	4.82±0.15	1.14±0.01	1.10±0.01	241±22	0.36±0.05	31±2
EXPERIMENT B								
Hypophysectomized								
Untreated	6	—	—	—	—	—	0.28±0.02	29±1
Growth hormone	3	485±19	5.94±0.36	1.24±0.03	1.29±0.03	321±22	0.36±0.05	31±2
Normal								
Untreated	6	—	—	—	—	—	2.22±0.10	129±7

[a] Mean ± standard error.
[b] See Table 1.
[c] Unilateral; consisted of quadriceps, hamstring, adductor, and gluteal muscles, related tendons, fascia, nerves, blood vessels, and fat.
[d] Unilateral; consisted of femur, tibia, fibula, and knee joint.

acts as a growth stimulant in hypophysectomized rats only by increasing their food intake.

The nature of the protein gain in hypophysectomized rats treated with growth hormone differs in several ways from that in hypophysectomized

conditions they retained 40 mg. of nitrogen/day. Without insulin they died within 48 hours.

TABLE 3. EFFECTS OF FORCE-FEEDING, INSULIN, AND GROWTH HORMONE ON THE COMPOSITION OF BODY AND BODY WEIGHT GAIN[a]

Groups[b]	No. of rats	Total carcass, gm.[c]		Gain, gm.[d]		
		Nitrogen	Fat	Body weight[e]	Protein (Nitrogen X6.25)	Fat
EXPERIMENT A						
Hypophysectomized						
Initial	7	3.52±0.14	20.2±1.3	—	—	—
Force-fed, untreated	8	3.97±0.10	41.6±2.5	32±2	2.7±0.6	21.2±2.0
force-fed, insulin	9	3.92±0.08	41.9±1.6	30±3	2.4±0.6	21.7±1.6
EXPERIMENT B						
Hypophysectomized						
Growth hormone	3	4.27±0.10	18.6±1.8	27±4	4.1±0.2	−1.6±2.1

[a] Mean ± standard error.
[b] See Table 1.
[c] Included all viscera; blood and gastrointestinal contents were discarded.
[d] Gain during experiment.
[e] Less the gain in weight of the gastrointestinal contents.

rats overfed or treated with insulin. When growth hormone is injected, protein is retained with a smaller food intake and the gain in protein is always greater than the gain in fat (Tables 1 and 3) (1, 8, 9). Without growth hormone the gain in fat is greater than the gain in protein (Table 3) (1, 3). Another difference is that growth, a weight gain with the same chemical composition as that of normal growing animals, has been sustained over a long period of time in hypophysectomized rats treated with growth hormone (10); such has not been observed in hypophysectomized rats overfed or treated with insulin (1, 3, 4).

In the force-fed hypophysectomized rats (Experiment A), the total amount of nitrogen retained as determined by the intake-output balance was greater than the gain in the nitrogen content of the carcass; 1300 mg./rat (Table 4) vs. 400 mg./rat (Table 3) (averages of insulin-treated and untreated combined). Although small losses probably occurred in the metabolic cages (by evaporation and spillage, etc.), and when the blood was discarded and the carcasses hydrolyzed with alkali, the major loss was most likely the hair which was shed by the rats and which was, unfortu-

TABLE 4. EFFECT OF INSULIN ON BODY WEIGHT GAIN AND NITROGEN RETENTION IN FORCE-FED HYPOPHYSECTOMIZED RATS[a]

Insulin, units/day	Food intake[b], gm./day	No. of days	Body weight gain, gm./day[c]		Nitrogen retained, mg./day[c]	
			Untreated (5)	Insulin-treated (6)	Untreated (5)	Insulin-treated (6)
2	7	3	0.6±0.4	0.6±0.3	36± 6	36±4
2	9	3	2.3±0.3	1.6±0.3	52±10	58±7
3	10	7	1.8±0.1	1.7±0.2	49± 4	60±4
3	11	9	1.9±0.1	1.8±0.1	59±11	71±7

[a] These rats, which were housed in individual metabolism cages, were part of experiment A (see Tables 1–3, and Fig. 1). There were 5 in the untreated and 6 in the insulin-treated group. The amount of nitrogen retained was determined as the difference between the amount fed and that excreted in the urine and feces.
[b] The average nitrogen content of the food was 28.4 mg./gm. of diet.
[c] Mean ± standard error.

nately, discarded. Starting the second week after hypophysectomy, these rats shed much more hair per day than rats with intact pituitaries maintained under the same conditions. In spite of their increased hair loss, an outstanding characteristic of these hypophysectomized rats was their luxuriant hair coats. It has also been observed by others that hair growth in this strain of rats is markedly accelerated by hypophysectomy (11). Since hair is nearly all protein, a loss of only 200 mg. of hair per day would account for most of the nitrogen lost; normal rats of the same size shed 70 mg. of hair per day (12). Further study of this problem is in progress. It is of interest to note that in young sheep maintained under conditions of severe undernutrition keratin synthesis continues at a normal rate, even at the expense of other tissue proteins (13).

SUMMARY

Hypophysectomized rats maintained their body weight when tube-fed 7 gm. of food daily. When the food intake was increased to 9–11 gm. they gained 32 gm. in 22 days. Although two-thirds of the weight gain was fat, there was a gain of 2.7 gm. in the protein content of the animals. Administration of 2–3 units of insulin daily had no effect on the body weight or composition of tube-fed hypophysectomized rats. In hypophysectomized rats treated with growth hormone, nitrogen retention occurred with a smaller food intake, 7 gm./day, and the gain in the protein content of the animals was greater than the gain in fat; without growth hormone the gain in fat was seven times greater than the gain in protein.

Acknowledgments

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THE LOCALIZATION OF THYROID HORMONES IN THE ORGANS AND TISSUES OF THE GUINEA PIG: AN AUTORADIOGRAPHIC AND CHROMATOGRAPHIC STUDY[1,2]

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THERE is little or no information available on the localization of thyroid hormones within organs and tissues. One short survey of the autoradiographic distribution of endogenously labelled thyroid hormone in the tissues and organs of the rat is included in a general survey of autoradiography (1) (See Fig. 78 in that article). Subsequent to this there have been reports on the localization of radioactivity in the pituitary and the central nervous system of the rabbit after treatment with labelled thyroxine (2) (3) and iodide (4). The results to be reported in this paper represent a preliminary survey by means of autoradiography of the localization of radioactive iodine in a variety of tissues and organs after administration of either radioactive thyroxine (T_4) or radioactive triidothyronine (T_3). The chemical composition of the concentrated radioactive materials was determined by concurrent chromatographic analysis. In this way a more precise insight into the localization of biologically active thyroid products was obtained.

MATERIALS AND METHODS

Animals. A total of ten guinea pigs weighing between 400 and 720 gms. were used in these experiments. The animals were obtained from Rockland Farms, New York City, New York. They were kept in a temperature-controlled animal room at 21° C ±2°. Their diet consisted of rabbit pellets supplemented with fresh greens and oats.

Materials. Thyroxine or triiodothyronine labelled with I^{131} (Abbott Laboratories) and dissolved in 50% propylene glycol was suitably diluted with normal saline, so that doses of about 1 mc./kg. could be administered in a volume of 4 ml. This amount of radioactivity was equivalent to an average of 38 μg. of thyroxine or 18 μg. of triiodothyronine. Four male animals received thyroxine, three males and one female received triiodothyronine, and one male animal received an equivalent dose of NaI^{131} (Oak Ridge). The remaining animal (male) was given no radioactivity, so that its tissues could be used to detect the possible formation of chemical artefacts in the course of the autoradiographic procedure.

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[2] Read before the American Goiter Association at the 1957 Annual Meeting in New York, N. Y.

Procedures. The animals were initially anaesthetized with barbiturates (dial-urethane or sodium evipal) and were kept anaesthetized during the period of injection by administration of ether. The labelled materials were injected into the exposed external jugular veins, the total dose being given in four equal divided doses at 15-minute intervals over a period of 45 minutes. Two hours after the last injection the animals were again anaesthetized and were perfused with citrated saline by means of a cannula tied into either the left ventricle or the aorta. The perfusion was carried on until the fluid escaping from a cut external jugular vein was completely free of blood. The tissues to be studied were then quickly removed and aliquots taken for autoradiography and for chemical analysis.

Autoradiography was carried out in the following way. After removal from the body, the tissues for this type of analysis were fixed in neutral 10 per cent formalin for from 24 to 48 hours. Previous work (to be published) has shown that formalin fixation causes the triiodothyronine present to be more firmly bound to the tissue proteins. The fixative leaches out only a small proportion of radioactivity, which the chromatogram shows to be iodide. After fixation, the tissues were placed in solutions of 10 and 20% gelatin in water for about 12 hours and then embedded in a 20% gelatin solution. The sections were allowed to dry and were then coated with celloidin (1%) in a solution of equal parts of ether and ethyl alcohol. The sections were placed in contact with no-screen x-ray film in x-ray film holders for periods of from 6 to 72 hours. Measurements of radioactivity at various stages of the procedure showed that no appreciable losses occurred. The illustrations (except for the picture of the ovary, Fig. 10) were taken from two representative animals (Guinea Pig 43, which received labelled thyroxine, and Guinea Pig 44, which received labelled triiodothyronine). The autoradiographs in all cases were exposed for 48 hours. Sections from the NaI^{131}-injected animal and the non-injected control were autoradiographically negative.

Since formalin fixation results in a firmer binding of triiodothyronine to the tissue protein, our usual method of chromatographic analysis could not be used on fixed tissues. Instead, aliquots of unfixed tissue were analyzed. The chemical analyses were carried out by single-dimensional chromatography in butanol-dioxane-ammonia of water homogenates of the fresh tissue and alkaline ethanol extracts of these homogenates. The procedure consisted in extracting a volume of homogenate with twice its volume of ethanol (made alkaline with ammonium hydroxide to a pH of about 8.5). Three further extractions were made, with equal volumes of alkaline ethanol. Over 90% of the total radioactivity was recovered by this procedure except in the case of the muscle from the triiodothyronine-treated animals, from which only 70% was recovered. The pooled extracts were reduced to a small volume under a vacuum at room temperature. In order more completely to identify some of the fractions found in the single-dimensional chromatograms, two-dimensional paper chromatograms of the extracts were prepared, butanol-dioxane-ammonia being used in the first dimension and tertiary-amyl alcohol ammonia in the second. In some cases Kieselguhr columns were used. In addition to scanning the strips, autoradiographs were made from them. The analyses are illustrated by the scans obtained with a gas-flow chromatogram scanner. The identification of apparently displaced peaks was confirmed by the additional analytical procedures and could be usually attributed to tailing, in the single dimension.

RESULTS

The results of the autoradiographic analyses are shown in Figures 2, 4, 6, 8, 10, 11, and 13. Corresponding scans of the chromatographic strips showing the radioactivity in these tissues are represented by Figures 1, 3, 5, 7, 9, 12, and 14.

The composition of the injected substances and their metabolism in the plasma during the course of the experiment is shown in Figure 1. In the case of thyroxine, the injected material contained a small amount of activity which showed up on the chromatogram in the iodide region (Fig. 1a). At the end of the experiment there was little change in the relative pro-

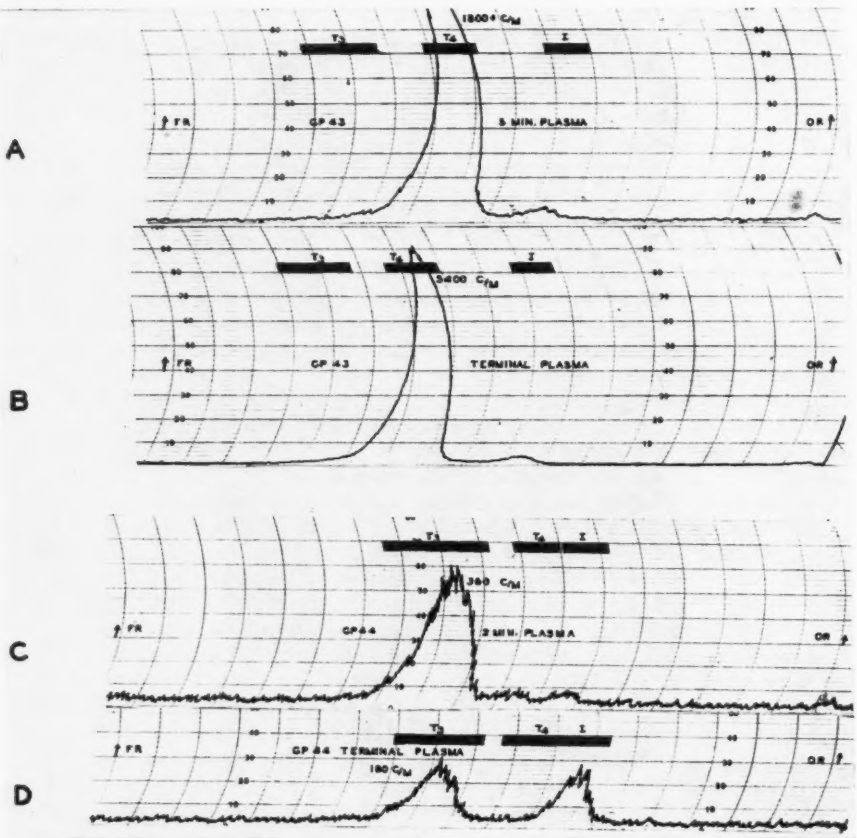


FIG. 1. Scan of the chromatographic analysis of initial and terminal plasmas from guinea pigs injected with I^{131}-labelled thyroxine [Guinea Pig (GP) 43] or triiodothyronine [Guinea Pig (GP) 44]. OR = Origin; FR = Front; T_3 = Triiodothyronine; T_4 = Thyroxine; I = Iodide.

portions of radioactivity present as thyroxine and as iodide (Fig. 1b). On the other hand, in the triiodothyronine experiment there was a relative decrease, as the experiment proceeded, in the proportion of the plasma radioactivity present as triiodothyronine. This is clearly seen by comparing the plasma analysis at 2 minutes after the injection (Fig. 1c) and at the

end of the experiment (Fig. 1d). These changes in plasma radioactivity will be used as a base line for the interpretation of the metabolites possibly responsible for the autoradiographic reactions in the tissues.

In general, for an equivalent dose of radioactivity and equivalent processing procedures, the autoradiographs of tissues containing triiodothy-

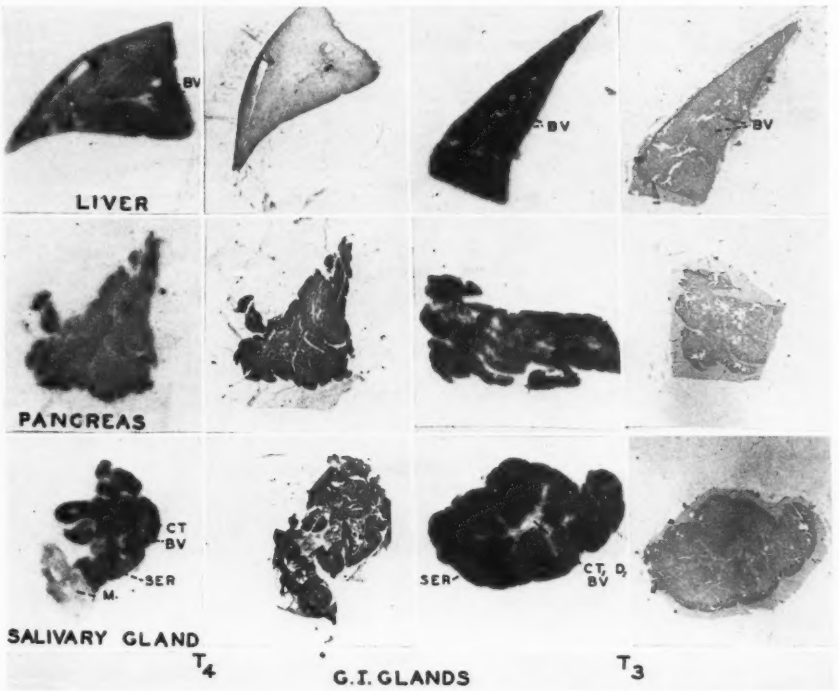


FIG. 2. Autoradiographic comparison of liver, pancreas and salivary gland from guinea pigs injected with I^{131}-labelled thyroxine or triiodothyronine. Enlarged 3×.

The two rows of figures at the left refer to Guinea Pig 43 (T_4), those at the right to Guinea Pig 44 (T_3). BV = Blood vessel; CT = Connective tissue; D = Salivary duct; M = Mucous gland cells, and SER = Serous gland cells.

ronine were considerably more intense and usually more distinctly localized than those of tissues containing thyroxine. This corresponds to the greater tissue uptake of triiodothyronine. The differences in distribution will be pointed out when the individual organs and tissues are examined.

The Gastrointestinal Tract

The glands of the gastronintestinal tract—the liver, the pancreas and the salivary glands—showed a diffuse reaction in the parenchyma (Fig. 2). The reaction over the pancreatic islets was of the same intensity as that

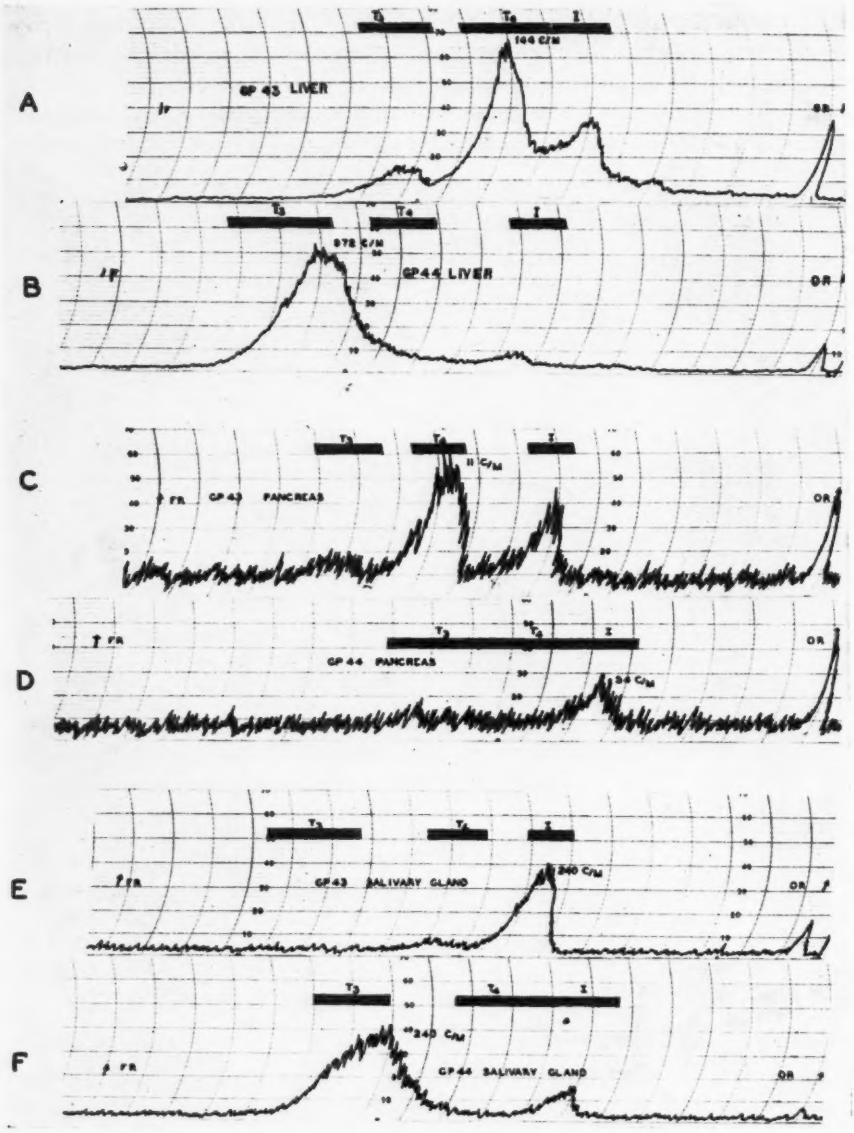


FIG. 3. Scan of chromatographic analyses of liver (A,B), pancreas (C,D) and salivary gland (E,F). The triiodothyronine observed in the liver homogenates from the thyroxine-injected animal was confirmed by two-dimensional paper chromatography and by column chromatography. OR = Origin; F = Front; T_3 = Triiodothyronine; T_4 = Thyroxine; I = Iodide.

over the exocrine portion of the pancreas. Similarly, the serous glands of the salivary gland showed a concentration of radioactivity which was greater after triiodothyronine than after thyroxine. The mucous-secreting part of the gland showed a much lower radioactivity after thyroxine. The radioactive localization was primarily in the glandular tissue in the case of both hormones; the reaction over the blood vessels, the connective tissue, and the ducts was weak in each case.

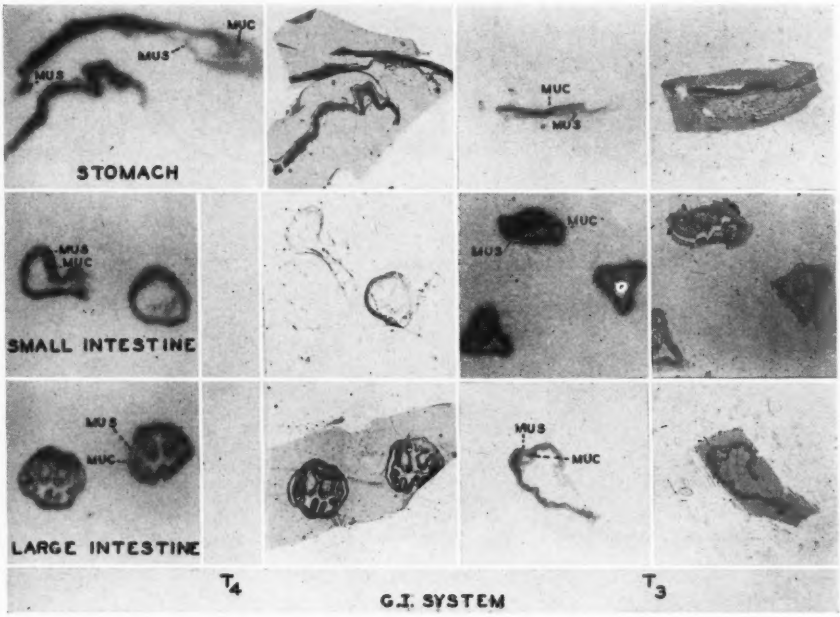
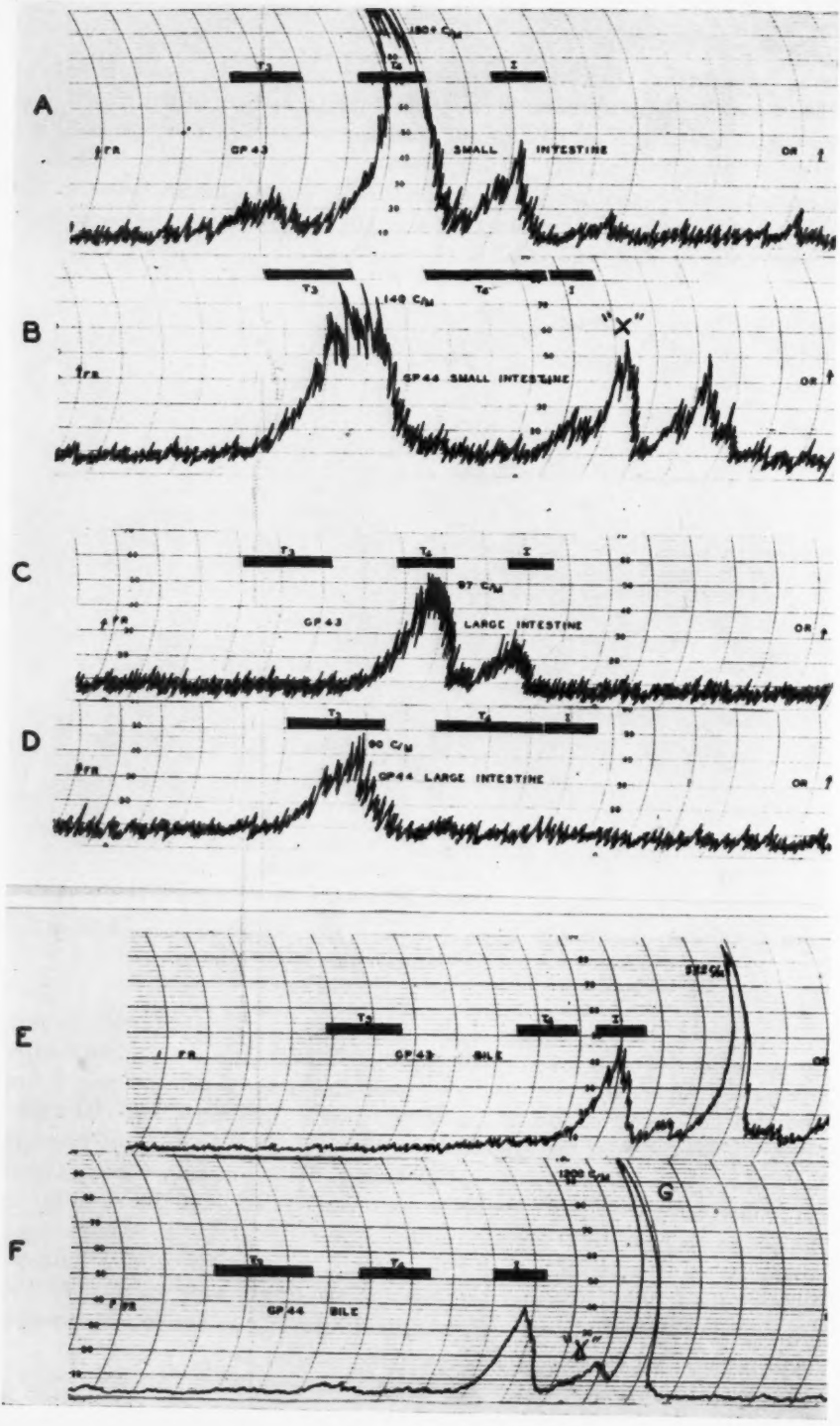


FIG. 4. Autoradiographic comparison of stomach, large intestine and small intestine. Enlarged 3×. MUS = Muscularis; MUC = Mucosa.

Analysis of these tissues (Fig. 3a, b, c, d, e, f,) indicated that the predominant radioactive constituent of the liver and the salivary gland after triiodothyronine injection was triiodothyronine. In the pancreas the major radioactive component appeared to be an iodinated material which did not migrate from the origin of the chromatogram and might possibly have been of a protein or polypeptide nature. With thyroxine, the analyses showed a somewhat more complex pattern. In the liver, in addition to thyroxine there were triiodothyronine, iodine, and a peak at the origin. A similar pattern was seen in the pancreas, but there, the relative proportions of the iodide peak and the peak at the origin were greater. In the salivary gland, the radioactivity was present mostly as iodide; there was a small peak at the origin.


A
B
C
D
E
F
G
GP 43
SMALL INTESTINE
GP 44 SMALL INTESTINE
"X"
GP 43
LARGE INTESTINE
GP 44 LARGE INTESTINE
GP 43
GP 44
BILE

The gastrointestinal tube, which had been washed free of contents, showed I^{131} localizations, which are illustrated in Figure 4. In the case of triiodothyronine there was clearly a concentration of radioiodine in the mucosa and a much weaker reaction over the submucosa and muscular coats. After thyroxine, however, there was a diffuse reaction throughout the wall of the organ and an equivalent concentration in the mucosa and the muscular coats. The over-all intensities in the stomach and large intestine were about equivalent for both substances, but there was a greater intensity in the small intestine of the triiodothyronine-treated animal. It is apparent from the chromatographic analyses in Figure 5 that the major iodine-containing compound in the walls of the small and the large intestine was thyroxine in the thyroxine-treated animal (Fig. 5a and c) and triiodothyronine in the triiodothyronine-treated animal (Fig. 5b and d). In the latter animal, the small intestine showed the presence of several iodine compounds migrating between iodide and the origin (Fig. 5b). Similar compounds were seen in the bile of both the triiodothyronine- and the thyroxine-treated animal (Fig. 5e, f). This would suggest that the small intestine can concentrate these derivatives of triiodothyronine but not similar derivatives of thyroxine. Alternatively, the mucosa in the small intestine may be able to form these derivatives metabolically from a triiodothyronine but not from a thyroxine substrate.

The Genito-Urinary System

The concentration of radioactivity in the kidney was greater after triiodothyronine than after thyrxone administration (Fig. 6). This strong reaction was localized in the cortex and was most distinct over the convoluted tubules. In somewhat less exposed preparations (not illustrated), it was possible to distinguish a weaker reaction over the glomeruli and the collecting tubules in the medullary rays. There was also a much weaker reaction over the medulla. By contrast, the kidney of the thyroxine-treated animal showed a concentration in the outer portion of the cortex which decreased continuously toward the medulla. In the cortex there was a weaker reaction over the collecting tubules of the medullary rays. The chromatographic analyses (Fig. 7a, b) showed that triiodothyronine was the predominant component in the triiodothyronine-treated animal and thyroxine in the thyroxine-treated animal. In the latter animal (Fig. 7a) there was also a considerable proportion of the activity present as tri-

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FIG. 5. Scan of chromatographic analyses of small intestine (A,B), large intestine (C,D) and bile (E,F). In B, note the compounds migrating between the origin and iodide. Peak labelled "G" in Figure 5F may be the glucuronide of triiodothyronine. Peak labelled "X" in Fig. 5B,F may be another triiodothyronine complex. OR = Origin; F = Front; T_3 = Triiodothyronine; T_4 = Thyroxine; I = Iodide.

iodothyronine, which undoubtedly contributed to the autoradiographic reaction.

With regard to the urinary passages (Fig. 6), the urinary bladder showed after treatment with thyroxine a marked concentration in the mucosal lining and a lesser reaction over the muscle. After triiodothyronine the concentrations of radioactivity in the mucosa and the muscle were of about

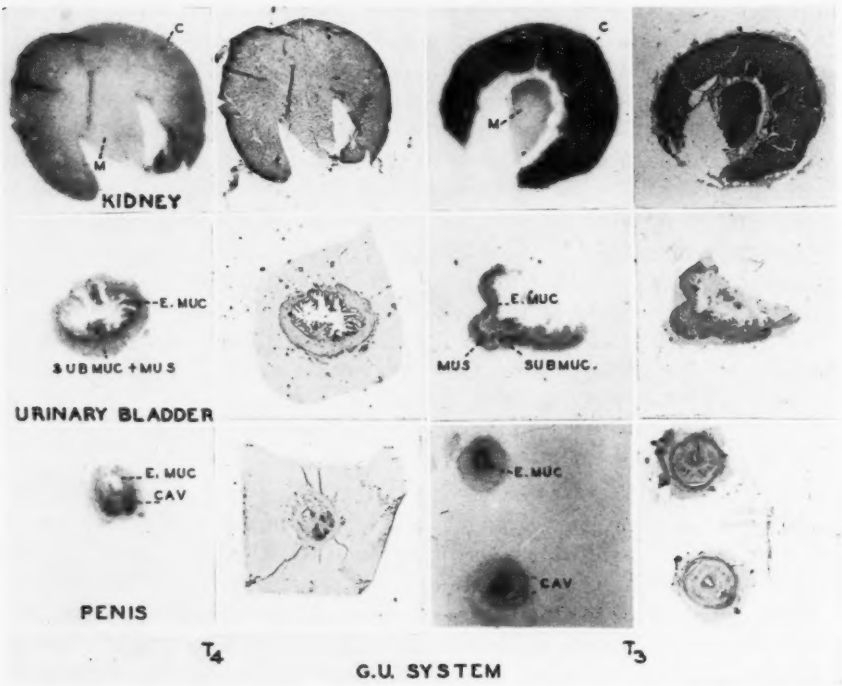

FIG. 6. Autoradiographic comparison of kidney, urinary bladder and penis. Enlarged 3×. C = Cortex; M = Medulla; MUS = Muscularis; E.MUC = Epithelial mucosal lining; SUBMUC = Submucosa.

the same intensity, but there was a considerably lesser reaction over the connective tissue of the submucosa. The penis of the thyroxine-treated animal showed a reaction over the poorly perfused blood-filled corpora cavernosa, but there was only a uniformly weak, diffuse reaction over the rest of the organ. The triiodothyronine-treated animal demonstrated a definite reaction in the mucous membrane lining the urethra, while the well-perfused corpora cavernosa were indistinguishable from the surrounding tissue. The chromatographic analysis for these tissues (Fig. 7c–e) would indicate that thyroxine and triiodothyronine were responsible for the autoradiographic reaction in the respectively treated animals. In

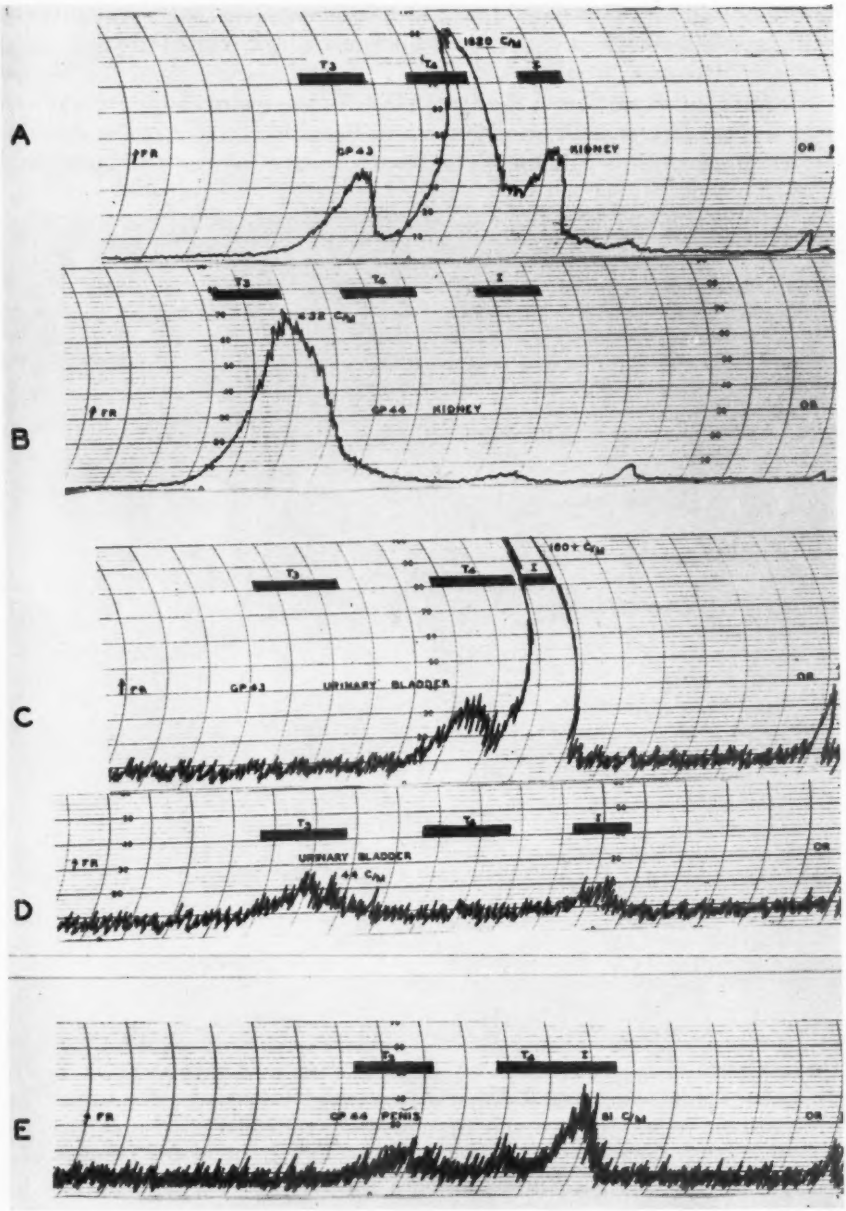

FIG. 7. Scans of chromatographic analyses of kidney (A,B), urinary bladder (C,D) and penis (E – T_3). OR = Origin; FR = Front; T_3 = Triiodothyronine; T_4 = Thyroxine; I = Iodide.

addition, consideration should be given to the possible participation of the iodinated substance present at the origin of the chromatogram (Fig. 7c–e).

In the genital portion of the tract (Fig. 8), the testes showed after both types of treatment a diffuse reaction corresponding to that of the seminiferous tubules. No evidence of greater concentration in the interstitial

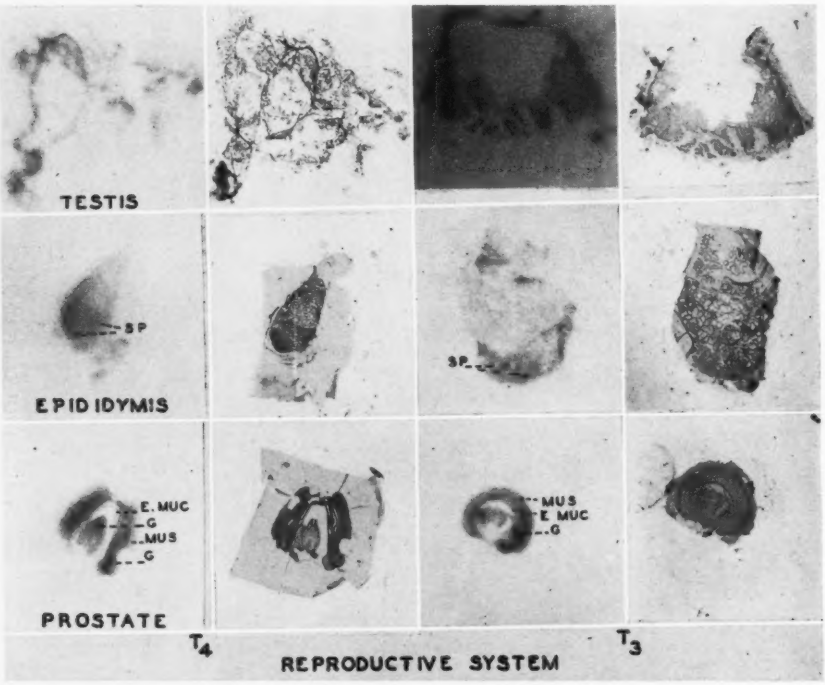

FIG. 8. Autoradiographic comparison of testis, epididymis and prostatic urethra. Enlarged 3×. SP = Sperm-filled tubules in the epididymis; G = Prostatic glands; MUS = Muscularis.

cells could be found. The autoradiographic reaction could be attributed to the presence of thyroxine and of triiodothyronine, respectively (Fig. 9c, d). In the epididymis (Fig. 8) there was a weak reaction over the tubules in both the thyroxine-treated and the triiodothyronine-treated animal. The reactions were stronger in areas where spermatozoa filled the lumens (see arrows, Fig. 8). The prostates showed a concentration of radioactivity in the glands (Fig. 8) and a weak but definite reaction in the associated urethral mucosa and muscle. One triiodothyronine-treated animal was female, and the localization of radioactivity in the ovary is shown in Figure 10. There was a definite concentration in the granulosal tissue

of the follicles and in the corpora lutea. The interstitial tissue was only weakly reactive, and the follicular fluid contained very little radioactivity. The radioactivity of these latter organs was too low to permit chromatographic analysis, and the chemical nature of the agent responsible for the autoradiographic reaction is at present uncertain. This is also true of the aorta (Fig. 10), which showed a weak, diffuse reaction through its wall,

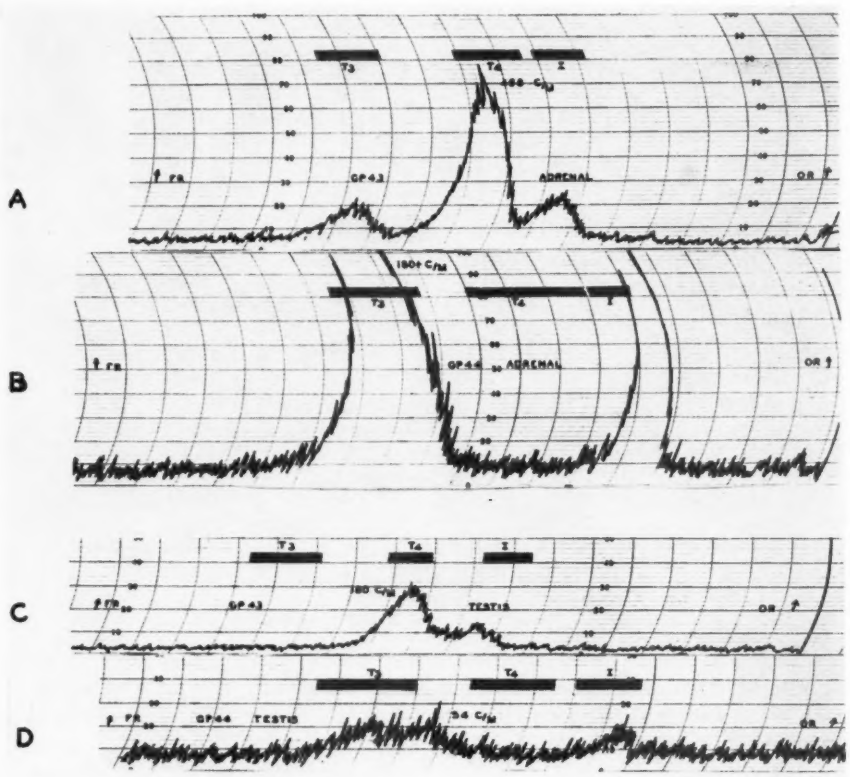

FIG. 9. Scans of chromatographic analyses of adrenal (A,B) and testis (C,D). OR = Origin; F = Front; T_3 = Triiodothyronine; T_4 = Thyroxine; I = Iodide.

and the gallbladder (Fig. 10), which showed a concentration in the epithelium.

Miscellaneous Tissues

The *adrenal* glands (Fig. 10) presented a strikingly different picture in the two autoradiographs. There was a weak, diffuse reaction over the cortex and the medulla after thyroxine, which was due mainly to the presence of thyroxine, though some triiodothyronine was present (Fig. 9a). In the

animal injected with triiodothyronine, there was a decided concentration of radioactivity over the cortex and a weaker but definite reaction over the medulla. The chromatogram (Fig. 9b) indicates that triiodothyronine was the predominant iodinated compound present, but it also suggests that another, unidentified, substance may contribute to the autoradiographic reaction.

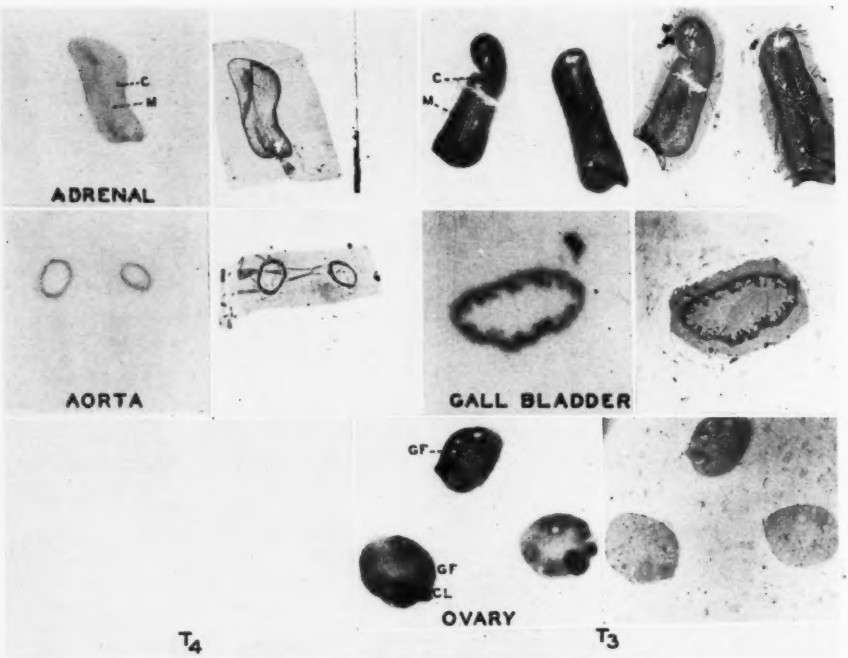

FIG. 10. Autoradiographic comparison of adrenal glands. Also included are aorta (GP 43) and sections of bladder and ovary (GP 44). Enlarged 3×. C = Cortex; M = Medulla; GF = Graafian follicle; CL = Corpus luteum.

The musculature of the body showed varying degrees of concentration (Fig. 11). The heart muscle produced an intense autoradiograph after injection of triiodothyronine, which was due entirely to that substance (Fig. 12b). The same results were seen in the case of skeletal muscle, except that the reaction was less strong (Fig. 12d). As may be seen in the autoradiograph (Fig. 11), the distribution of radioactivity corresponded almost precisely to the muscle bands. That radioactivity can enter smooth muscle to some extent is shown in autoradiographs of the muscular wall of the intestinal tube (Fig. 4). After thyroxine treatment the concentration of radioactivity in the heart and skeletal muscle was very much weaker than after triiodothyronine. In the skeletal muscle an ample proportion of the

radioactivity present was in the form of thyroxine (Fig. 12c), while in the heart muscle the relative proportion of thyroxine present was low (Fig. 12a).

In the *skin*, triiodothyronine (Fig. 12f) localized most strongly in the hair follicles (Fig. 11); there was a lesser concentration in the outer layers of skin, and the lowest concentration was in the dermis. A similar but much weaker

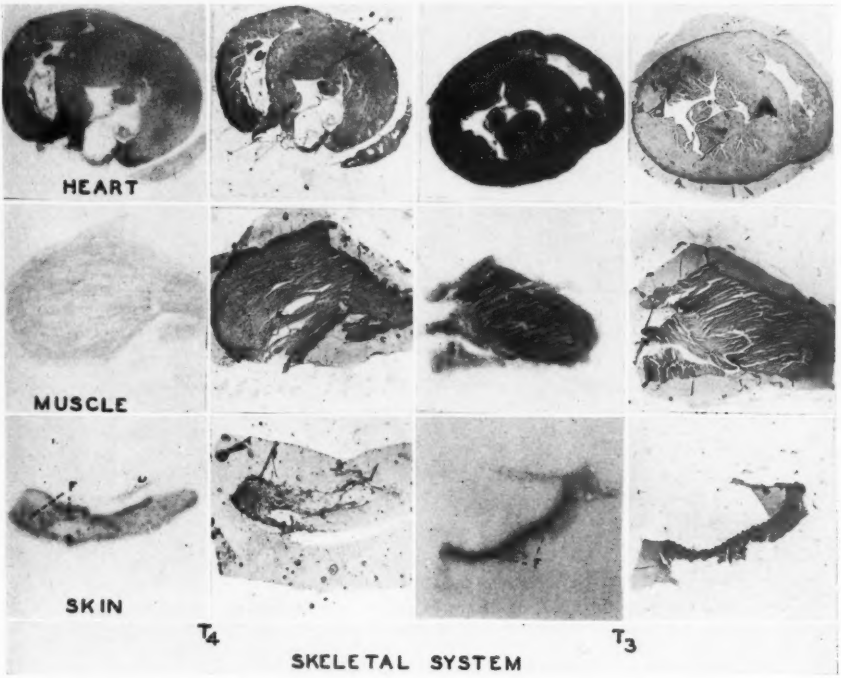

FIG. 11. Autoradiographic comparison of heart, pectoral muscle and skin. Enlarged 3×. F = Hair follicle.

reaction was seen after thyroxine treatment. Analysis of tissue from animals so treated (Fig. 12e) showed a large proportion of I_{131} as iodide and relatively little as thyroxine or as activity at the origin.

The *lymphatic organs*, the spleen and the lymph nodes, showed a generalized, diffuse distribution of radioactivity after thyroxine (Fig. 13). In the spleen there were a few areas of concentration corresponding to the *red pulp*. The area of greatest density in the lymph node corresponded to the location of a lymph nodule. In spleen and, lymph node, radioactive thyroxine could be demonstrated (Fig. 14a, c), but in the case of the lymph node it was proportionally exceeded by radioactive iodide. After triiodothyronine, both the spleen and the lymph node showed a denser reaction

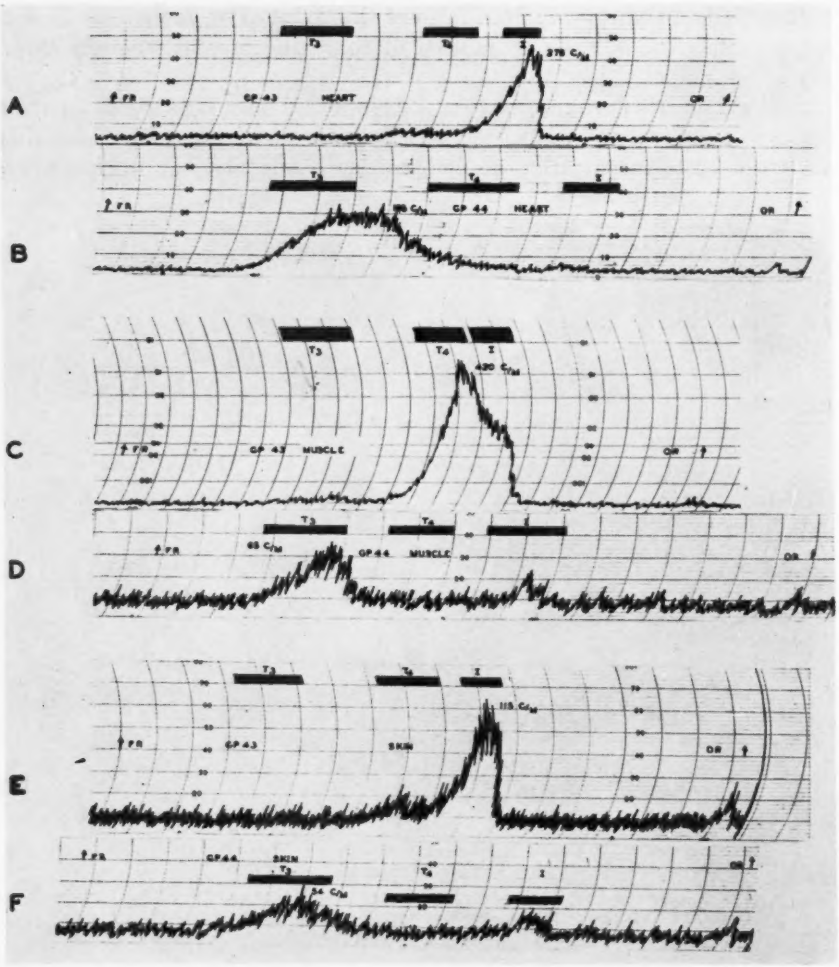

FIG. 12. Scans of chromatographic analyses of heart (A,B), pectoral muscle (C,D) and skin (E,F). Note in B that the single dimensional chromatographic peak for triiodothyronine tailed. The identity of the material in this area as triiodothyronine was confirmed by two-dimensional paper chromatography. OR = Origin; F = Front; T_3 = Triiodothyronine; T_4 = Thyroxine; I = Iodine.

than after thyrxoine (Fig. 13). In the triiodothyronine-treated animal, random areas of strong concentration (hot spots) were noted in the red pulp, but these could not be related to specific minute structures by means of the relatively crude resolution of the autoradiographic technique. That the autoradiographs of these tissues were due primarily to their triiodothyronine content is shown in the analysis in Figure 14 (b, d).

The *lung* showed a weak, diffuse reaction after thyroxine treatment (Fig. 13). An occasional area of concentration in the parenchyma was noted in all sections. These areas did not correspond either to the bronchi or to the vessels. From Figure 14e it is likely that the autoradiographic pattern is due to radioactive thyroxine. After injection of triiodothyronine the concentration was more pronounced, and it was possible to see that the autoradiograph corresponded to the alveolar tissue and that the reac-

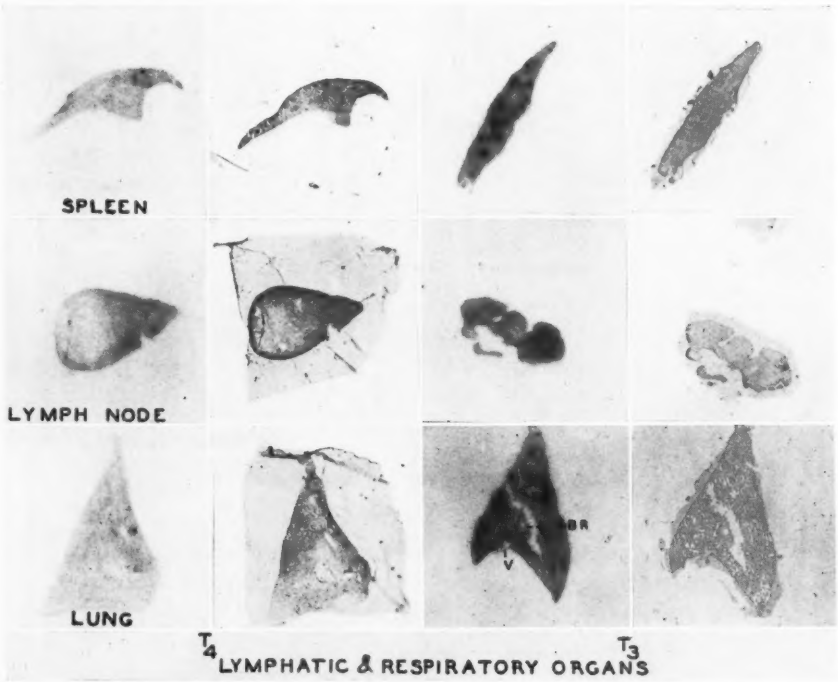

FIG. 13. Autoradiographic comparison of spleen, lymph node and lung. Enlarged 3×. BR = Bronchiolar tube; V = Vein.

tion over the bronchi and blood vessels was very much less intense. Areas of concentrated radioactivity occurred at random throughout the parenchyma. These were consistently present and occurred more frequently than in the lung of the thyroxine-treated animal. From the chromatographic analysis in Figure 14f, it is probable that lung radioactivity represents the presence of labelled triiodothyronine. It is possible that the hot spots noted in the spleen and the lung may be due to accumulations of reticuloendothelial cells which have ingested radioactive tissue components.

While the brains and the pituitaries of the animals were also examined, the results will be given in detail in a separate publication. For complete-

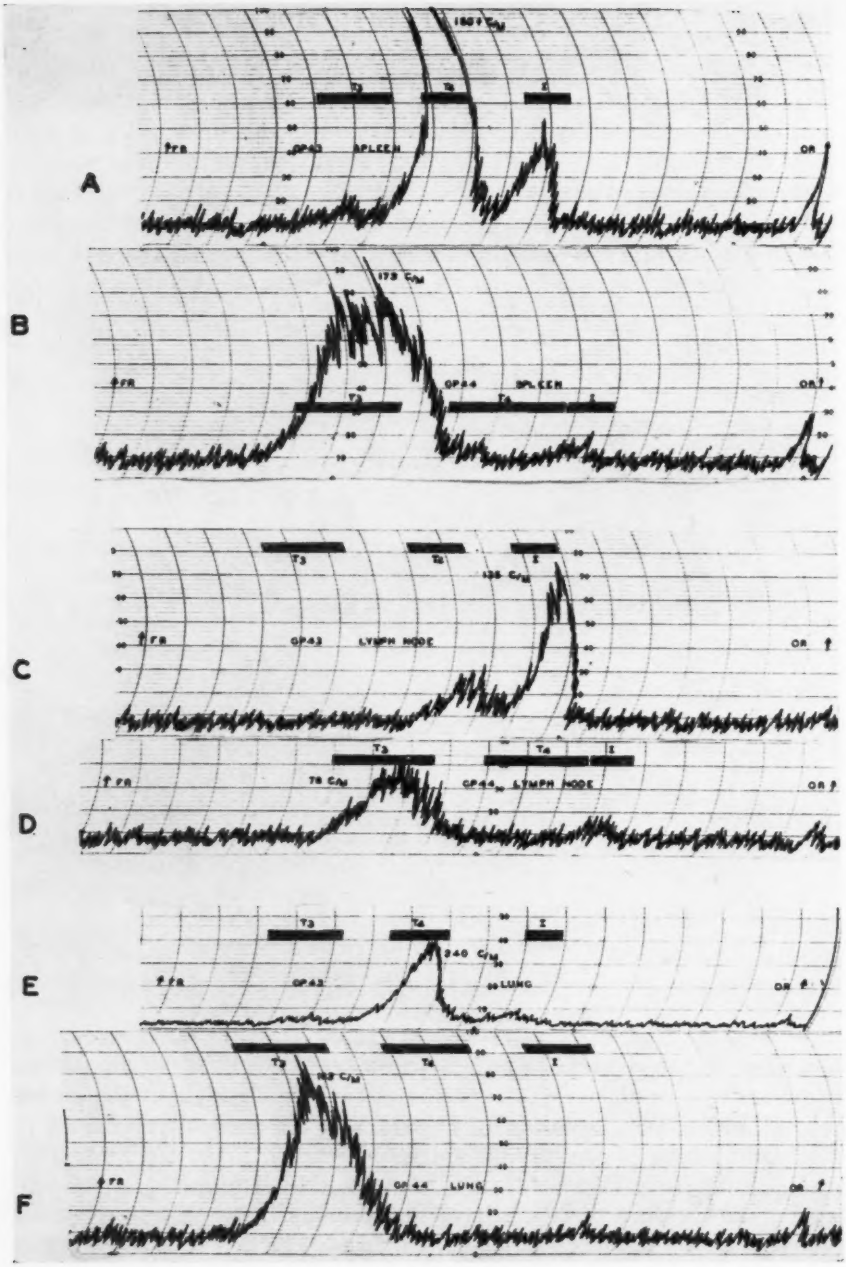

FIG. 14. Scans of chromatographic analyses of spleen (A,B), lymph node (C,D) and lung (E,F). Note in B, the triiodothyronine peak showed tailing and was confirmed as triiodothyronine by repeat chromatography. OR = Origin; F = Front; T_3 = Triiodothyronine; T_4 = Thyroxine; I = Iodide.

ness, it may be noted that concentrations of triiodothyronine were found in the anterior pituitary, the posterior pituitary, and the area of the hypothalamus which contains the paraventricular and supraoptic nuclei. Thyroxine-treated animals showed virtually no concentration in these areas of the hypothalamus.

DISCUSSION

The autoradiographic distribution of radioactivity in tissues after triiodothyronine administration would seem to give the simplest pattern of localization of the thyroactive substances. This is suggested by the fact that, except in the pancreas and the urinary passages (Figs. 3d and 7d, e), triiodothyronine is the predominant iodine compound present.

The pattern and degree of this localization may be associated with the degree of metabolic activity (QO_2) occurring in the the cells. Note the parallelism between the descending degree of concentration in the following organs and the accompanying figures (taken from Spector (5)) representing metabolic activity: kidney cortex ($QO_2 = 31.8$), the liver ($QO_2 = 13.0$), the lung ($QO_2 = 8.5$), the spleen ($QO_2 = 11.6$) and smooth muscle ($QO_2 = 1.7$). This would also be consistent with the density of the autoradiograph over those areas of the epididymus which contained sperm, since guinea pig sperm shows a QO_2 of 8.0 (5). Similarly in the spleen, the greater concentration in the red pulp as compared with the white pulp or the lymphatic tissue might be associated with a greater metabolic activity. This is supported by data on oxygen consumption in the rat spleen (5), which show that the QO_2 of total spleen is more than twice as high as the QO_2 of lymphatic tissue itself.

Possibly related to specific metabolic activity, there is an obvious concentration in organs with active secretory capacities. This is made evident from the localizations found in the adrenal, the salivary glands, the secretory tissue of the ovary, the gallbladder, and the intestinal mucosae. The exception to this generalization is the lack of a compartively strong concentration in the interstitial cells of the testis.

The concentrations in the mucosa of the gastrointestinal tract and in the hair follicles (and to a lesser extent the outer, possibly epidermal, layers) of the skin might suggest an association of thyroid hormone with sites of active cell division. This might account for the diffuse reaction over the seminiferous tubules and the weak localization found in the lymphatic nodules of spleen and lymph node. It may be further pointed out that the small intestine of the triiodothyronine-treated animal is decidedly more reactive than the large intestine, which provides a parallel to the gradients of cellular activity and the cell replacements of the two structures.

In the thyroxine experiments, the analytical data indicate the presence of some thyroxine in all the perfused tissues examined. This would imply the entry of thyroxine into tissues. The autoradiographs show a qualita-

tive distribution similar to that obtained in the triiodothyronine-treated animal, but this may be due to the presence of metabolites of thyroxine in addition to the compound itself. (See below.) Nevertheless, these results indicate that while there are evident quantitative differences, both triiodothyronine and thyroxine can gain access to similar tissual sites. This would indicate that thyroxine does not have to be converted to triiodothyronine in order to enter tissues. This is contrary to a suggestion previously made by one of the authors (6), which was based solely on the relative quantitative concentration of thyroxine and triiodothyronine in tissues.

The autoradiographs obtained after the administration of thyroxine present some difficulties in interpretation because of the complexity of the metabolites present in the tissues. In all the organs analyzed, the proportion of the radioactivity present as iodide greatly exceeded the proportion present in the plasma throughout the course of the experiment (Fig. 1a,b). In some organs (salivary glands, heart, skin, lymph node, and urinary bladder) iodide was the predominant constituent of the tissual radioiodine. Since in the control experiments it was found that there was no concentration of injected iodide, it is likely that the iodide fraction demonstrable in the tissues after the administration of thyroxine is intracellular and represents the result of the local deiodinase activity of the cells, and that the equilibration of intracellular and extracellular iodide occurs rather slowly. It is possible that the iodide fraction contributed to the production of the autoradiograph and would account for the more diffuse localizations obtained in the thyroxine-treated animal. Proof of this assumption must await direct analysis of the fixed tissue. The slow rate of equilibration of iodide is also illustrated in the results of the triiodothyronine experiments. Here, despite the steadily increasing proportion of radioiodide in the plasma (Fig. 1c,d), the relative proportion of iodide in the tissues (except in the pancreas) is rather low. If this reasoning is right, then the higher iodide proportion after thyroxine treatment would indicate that the tissual deiodination of thyroxine is more rapid than that of triiodothyronine (except in the pancreas).

In this connection it should be noted that triiodothyronine was demonstrable in a number of tissues of the thyroxine-treated animals. These include liver, pancreas, small intestine, kidney and adrenal. There are three possible sources of this fraction: (1) It may have been present in the injection mixture. If so, the amount present must have been very low, since it was not clearly detectable as a peak in the analysis (Fig. 1a). (2) The triiodothyronine may have been formed in the thyroid and secreted into the circulation. The low thyroid uptake (.5%) and the short duration of the experiment would tend to argue against this possibility. (3) It may have been formed within the respective organs as a metabolite of thyroxine. This would be a logical intermediate step in the apparently active deiodina-

tion of thyroxine in the tissues. It has been shown to occur in isolated kidney tissue (7) and in the perfused kidney *in vitro* (8). Keeping in mind that the triiodothyronine content of some organs after administration of thyroxine may have been due to their concentration of triiodothyronine which had been formed in other organs, it is an interesting speculation that some of the thyroxine autoradiographs may also indicate the sites of peripheral triiodothyronine formation. The present state of knowledge permits this speculation only with respect to the kidney, with the suggestion that triiodothyronine formation may occur in the outer cortex of this organ.

The unidentified iodine compounds detected in some of the tissues require some comment.

The bile (Fig. 5e, f) shows the presence of several substances which are being secreted into the lumen of the small intestine. Substances with a similar chromatographic location were found in the wall of the small intestine of the triiodothyronine-treated animal (Fig. 5b) but not in the intestine of the thyroxine-treated animal (Fig. 5a). This would imply that these metabolites of triiodothyronine are either more readily absorbed or that they are more readily formed in the small intestine than similar metabolites of thyroxine. The nature of these substances was not determined, but from previous work it is thought likely that the compound labelled "G" (Fig. 5f) is the glucuronide of triiodothyronine (9), and it is possible that the compound labelled "X" (Fig. 5b,f) is another triiodothyronine complex (10).

The nature and the significance of the material occurring at the origin of the chromatograms of some tissues, e.g., liver and pancreas (Fig. 3a, b, c, d), is not clear at the present time. It is a possibility that this material may be of a protein or polypeptide nature.

During the course of the analytical work, attempts were made to identify the acetic acid analogues of the substances injected. Except in the case of the terminal plasma of the triiodothyronine-treated animal, which contained a small amount of triiodothyroacetic acid, no triiodothyroacetic (TRIAC) nor tetraiodothyroacetic (TETRAC) acids were found in any of the other tissues examined, despite the separation of sizeable amounts of tissue extract on Kieselguhr columns and the subsequent concentration and two-dimensional paper-chromatographic analysis of the fractions. This is at variance with reports of the presence of TRIAC in the muscle and kidney of triiodothyronine-treated rats (11). In the rat experiments the tissues had not been perfused prior to study, and it is possible that the TRIAC found in these tissues was present in plasma that remained with the organ after excision. On the other hand, the perfusion procedure in the guinea pig experiments may have removed the material. This latter would seem, however, an unlikely possibility in view of the failure of the perfusion procedure to remove from the tissues such small molecules as iodide.

SUMMARY

Guinea pigs were injected with I^{131}-labelled thyroxine or triiodothyronine. After perfusion, the tissues were analyzed by contact autoradiography for the distribution of radioiodine and by chromatography for the chemical nature of the tissual iodine.

After triiodothyronine, autoradiographs demonstrated the presence of radioiodine in the cortex of the kidney, the parenchyma of the liver, the alveolar tissue of the lung, the red pulp of the spleen, and cardiac, skeletal and smooth muscle fibers; in the following secretory tissues: adrenal cortex, ovarian granulosal and luteal tissues, serous tissue of the salivary glands, serous and acinar tissues of the pancreas, and the prostate; and in the mucosae of the stomach, the small and large intestines, the gallbladder and the urinary passages. Radioactivity was also detected in the hair follicles of the skin and, to a lesser extent, in its outer layers. Some evidence indicated its presence in the sperm.

From the chromatographic analyses, the radioactivity detected was chiefly triiodothyronine except in the pancreas, where it was found to be iodide and an unidentified iodine-containing compound.

After thyroxine, a similar but considerably less intense and more diffuse localization was demonstrable. The chemical analyses showed that a large fraction of the radioactivity was present as iodide. This fraction predominated in the salivary gland, skin, lymph node and urinary tract and would suggest active deiodinase activity. In addition, triiodothyronine was found in liver, kidney, pancreas, small intestine, adrenal and spleen, which in some of these tissues may represent an intermediate product of the deiodination process.

Small amounts of unidentified iodine compounds were detected. One of these, which occurred at the origin of the chromatograms, was found in the liver, pancreas, kidney, urinary bladder, lung, spleen and skin after both thyroxine and triiodothyronine administration. In the small intestine of the triiodothyronine-treated animals, substances were found which had a chromatographic location similar to that of some of the biliary iodine compounds. This was not seen in the thyroxine-treated animals. A small amount of triiodothyroacetic acid was detected in the terminal plasma of the triiodothyronine-treated animal. Efforts to identify either triiodoacetic or tetraiodoacetic acids in other samples were unsuccessful.

The demonstration of thyroxine in tissues indicates that its entry into possible target sites of thyroid hormone activity does not require its prior conversion to triiodothyronine.

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PATHWAYS OF THYROIDAL PHOSPHORUS METABOLISM: THE EFFECT OF PITUITARY THYROTROPIN UPON THE PHOSPHOLIPIDS OF THE SHEEP THYROID GLAND[1,2]

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IN THIS laboratory, several hypothetical considerations have prompted a sustaining inquiry into the intermediate metabolism of thyroid tissue. First, since hormonal biosynthesis may be delimited by intrathyroidal energy metabolism (1, 2, 3), the implications for thyroidal disease mechanisms are obvious. Secondly, since the preformed stores of thyroid hormone confer a unique "hyperthyroxinosis" upon thyroid tissue (4, 5, 6), it is tempting to speculate that the studies may aid in the elucidation of thyroid hormone action. Finally, since the multiple effects of pituitary thyrotropin (TSH) upon thyroid tissue have not been explained satisfactorily, it is hoped that such investigations may disclose a unifocal biochemical site of TSH action to which the other phenomena are related secondarily.

In the preceding communication (7), the phospholipids and the water-soluble phospholipid intermediates of sheep thyroid tissue have been identified and their metabolic relationships have been characterized *in vitro* by newly developed techniques (8, 9). The action of pituitary thyrotropin upon the phospholipid metabolism of thyroid tissue has been examined in the present study. Intact sheep and surviving sheep thyroid slices have been employed. The results confirm the preliminary observations of Morton and Schwartz that TSH *in vitro* augments the incorporation of P^{32} into unfractionated thyroidal phospholipids (10), and extend the more detailed

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investigations which have been reported in abstract form by Florsheim, Moskowitz, and Morton (11), and by Freinkel (12).

MATERIALS

Radioactive orthophosphate was obtained from the Radiochemical Centre, Amersham, England. Four different preparations of pituitary thyrotropin were employed: Armour "Thytropar" N2211, M2105, P2308 and IRW. The first three preparations of TSH were obtained commercially; Lot IRW of bovine thyrotropin, and Lot #732–181 of bovine somatotropin, Lot #264–151X of porcine follicle stimulating hormone, and Lot #759–39CCC of ovine prolactin were generously supplied by Dr. S. L. Steelman of the Biochemical Research Department, The Armour Laboratories, Chicago, Illinois. Corticotropin was kindly provided by Dr. H. B. F. Dixon, of the Department of Biochemistry, University of Cambridge, England. Thyroid glands were removed from sheep, one year of age or older, within five minutes of the killing of the animals.

METHODS

1. *In vivo study*: Four male sheep of comparable age, previous dietary and environmental exposure were each given 5 mc. of inorganic P^{32} intravenously. Two of the animals had received 10 U.S.P. units of TSH (Armour, Lot #P2308) intravenously three hours earlier. One TSH-treated animal and one untreated animal were sacrificed two hours following the administration of P^{32}. The remaining two animals were killed two hours later. Excised thyroid glands were directly plunged into liquid oxygen for storage prior to fractionation.

2. *In vitro studies*: Thyroid glands were wrapped in cellophane bags and transported to the laboratory on cracked ice contained within Dewar flasks. Control *in vitro* experiments were performed with sections of liver removed from the same animals and with portions of lactating rat mammary glands obtained as described previously (13).

Studies with surviving tissue slices were conducted in the Warburg apparatus at 38° C using pure O_2 in the gas phase. All incubations were started within less than two hours of the killing of the donor animals. Details of slice preparation, incubation and manometry have been outlined in previous reports (3, 14). Weighed amounts of tissue slices (150 to 350 mg. wet weight) were introduced into the main compartment of Warburg vessels which contained 2.7 ml. of a "phosphate-free" medium of the following composition: 0.131 *M* NaCl, 0.005 *M* KCl, 0.0012 *M* $MgSO_4$, 0.0008 *M* $CaCl_2$ and 0.010 *M* Tris (hydroxymethyl) aminomethane buffer (pH 7.4). For any single experiment, the wei hts of slices in the individual vessels agreed within ±10 mg. Sidearms of Warburg vessels were filled with 0.3 ml. of "phosphate-media" of similar ionic composition save for the substitution of 0.010 *M* sodium phosphate buffer (pH 7.4) for the Tris (hydroxymethyl) aminomethane. The side-arm solutions also contained 3 to 75 μc. of $NaH_2\ P^{32}O_4$ and either 0 or 1.0 U.S.P. units of TSH. Weighed amounts of lyophilized thyrotropin were freshly dissolved in the "phosphate-media" just prior to the filling of the side-arms. The same dose of TSH (i.e. 1.0 U.S.P. unit) was employed for all experiments. Preliminary studies had disclosed that this was slightly greater than the amount required to effect a plateau value of maximal thyrotropic stimulation while higher doses (i.e. 5 to 10 U.S.P. units per vessel) occasionally effected a depression.

The use of separate "phosphate-free media" in the main compartment and "phosphate media" in the side-arm facilitated certain experimental objectives. It permitted the introduction of inorganic P^{32} of known specific activity as well as TSH at desired times following the variable preparative delays. Thus, the duration of TSH action could be precisely documented, and the organic radioactivity of the slice at any single instant could be directly converted into a minimal estimate of the net incorporation of inorganic

phosphorus during a given interval by dividing the observed counts by the initial specific activity of the "phosphate media" in the side-arms. Following 1 to 4 hours of incubation, the slices were removed from the Warburg vessels, quickly blotted, reweighed on a torsion balance and frozen in liquid oxygen for storage prior to fractionation.

3. *Fractionation of tissues*: Water-soluble and lipid extracts of tissue phosphorus were prepared in a cold room maintained at 4° C in order to minimize the decomposition of organic phosphates. Two fractionation schema were employed. Thyroid glands from the *in vivo* experiment and slices from five of the *in vitro* studies (expt. #8–12) were fractionated by the modified techniques of Dawson (8, 9) which have been described elsewhere (7). For other studies, frozen slices from individual vessels were homogenized with 3 ml. of chilled 10% trichloracetic acid (TCA) in a ground-glass, Potter-Elvehjem homogenizer. The homogenate was centrifuged and the supernatant was removed. The precipitate was washed four additional times with 5 ml. of portions of 5% TCA. The initial supernatant and all subsequent washings were pooled and diluted to constant volume. Lipids were removed from the washed, TCA-insoluble material by treating it twice with 3 ml. portions of methanol, twice with 5 ml. portions of boiling 3:1 methanol:ether (v/v), and once with 5 ml. of diethyl ether. The organic extracts were combined and diluted to 25 ml. in a volumetric flask. Control studies were performed in which portions of the same thyroid gland were fractionated by the two techniques. Significant differences in the recovery of total lipid phosphorus could not be demonstrated. Further control experiments indicated that contamination with inorganic P^{32} from the medium could not have accounted for more than 0.7% of the total radioactivity which was recovered, in lipid extracts prepared by either technique of fractionation.

In three experiments, water-soluble phosphorus was partitioned into inorganic and organic components by the isobutanol-extraction procedure of Ennor and Stocken (15). Individual water-soluble compounds were isolated by chromatographing aqueous extracts in two dimensions on paper as has been described previously (9, 7). In six *in vitro* studies and in the *in vivo* experiment, the individual phospholipids in lipid extracts were examined by the mild alkaline hydrolysis and paper chromatographic techniques of Dawson (8). When methanol-and-ether lipid extracts were employed for mild alkaline hydrolysis, they were preliminarily shaken with 0.8 volumes of chloroform and 2 volumes of 0.1N HCl, and the final chloroform phase was analyzed.

4. *Phosphorus analyses and radioactive assay*: Liquid aliquots were counted directly with a M-6 liquid-counter tube (20th Century Electronics) or plancheted onto aluminum discs, dried at room temperature, and counted at infinite thinness with a mica-end window Geiger-Mueller tube. Sufficient counts were observed to reduce the probable error of the measurement to less than 3%.

Total phosphorus in the water-soluble and lipid fractions was estimated by evaporating aliquots to dryness, digesting them on a microKjeldahl digestion rack with 0.9 ml. of 72% perchloric acid (A.R.), and analyzing the digests by the method of Fiske and Subbarow (16). Inorganic phosphorus in the isobutanol extracts of Ennor and Stocken (15) was directly measured by the method of Berenblum and Chain (17). Organic phosphorus in the water-soluble fraction was calculated as the difference between the measured values for total and inorganic phosphorus. Methods for the localization and the analysis of the individual phosphorus-containing components in chromatograms have been reported elsewhere (7).

In the *in vivo* experiment, specific activities were expressed as counts per minute per mg. P. For *in vitro* studies, all specific activities were expressed as counts per minute per μg. P and were corrected to an initial specific activity of 100,000 counts per minute per μg. P in the "phosphate-media" contained within the side-arms of Warburg vessels.

5. *Statistical analyses*: Statistical analyses were performed according to methods described by Kendall (18). Tests for significance of differences between means and for

correlation coefficients were based upon the appropriate modifications of the "t" test of "Student."

RESULTS

1. *Labelling of thyroidal phospholipids and water-soluble phospholipid intermediates in vivo:* Fractionation of the four thyroid glands revealed that the specific activities of the individual phospholipids varied considerably (Table 1). At 2 as well as at 4 hours following the administration of P^{32}, the phosphoinositide was labelled to a greater extent than any other lipid

TABLE 1. THE INCORPORATION OF P^{32} INTO THE INDIVIDUAL PHOSPHOLIPIDS AND PHOSPHOLIPID-INTERMEDIATES OF THE SHEEP THYROID GLAND *In Vivo*

	Specific activity (counts P^{32}/mg. P)			
	2 hrs. after P^{32}		4 hrs. after P^{32}	
	Control	+ TSH*	Control	+ TSH
Phospholipid type:†				
Phosphatidylcholine	500	1,700	800	4,300
Phosphatidylethanolamine	700	5,100	1,500	5,400
Phosphatidylserine	800	1,800	5,500	7,200
Phosphoinositide	9,700	18,500	12,500	29,800
Water-soluble intermediates:				
Phosphorylcholine	14,900	35,800	21,800	35,600
Phosphorylethanolamine	22,400	26,400	22,600	29,200
Glycerylphosphorylcholine	—	—	600	1,500
Glycerylphosphorylethanolamine	—	—	650	3,100

* TSH: 10 U.S.P. units given intravenously 3 hours before P^{32}.
† Hydrolysis products corresponding to phosphatidic acid could not be demonstrated in this experiment.

component. Although the phosphoinositide constitutes only about one-tenth of the lipid phosphorus of the sheep thyroid (7), it accounted for forty per cent or more of the total lipid radioactivity in these glands. The water-soluble phosphomonoesters, phosphorylcholine and phosphorylethanolamine, were more heavily labelled at 2 and at 4 hours than the corresponding phospholipids, phosphatidylcholine and phosphatidylethanolamine, whereas at 4 hours the specific activities of the phosphodiesters, glycerylphosphorylcholine and glycerylphosphorylethanolamine, were less than the phospholipid values (Table 1). The phosphodiesters were insufficiently labelled at 2 hours for accurate radioactive assay. In the TSH-treated animals, all of the phosphorus-containing compounds exhibited higher specific activities (Table 1).

2. *The effect of TSH upon thyroidal phospholipids and respiratory metabolism in vitro:* Results of twelve experiments with incubations varying from 1 to 4 hours are listed in Table 2. The average weight loss of thyroid slices incubated in the presence of 1.0 U.S.P. units of TSH was 15.5 ± 3.0[4]% of the original wet weight as compared to 18.9 ± 3.2[4]% in the control tis-

[4] Mean ± Standard Deviation of the Mean.

sues. The differences could be entirely attributed to variation in hydration of the tissues (19). Although not statistically significant for the group as a whole ($.15 < p < .20$), the differences were of sufficient magnitude in isolated instances to necessitate calculation of phosphorus incorporation in terms of initial as well as final wet weight.

In the sixteen observations of the twelve experiments, net incorporation of phosphorus into the phospholipids of control tissue showed a significant linear relationship to duration of incubation (correlation coefficient 0.882, $p < .001$) (Table 2). When the phosphorus incorporation into phospholipids of control tissues was corrected for time, a statistically significant relationship to resting QO_2 could also be demonstrated (partial correlation coefficient 0.556; $.01 < p < .05$). In every experiment, TSH effected an augmented incorporation of phosphorus into total thyroidal phospholipids. The stimulatory effects at the 1, 2, 3, and 4 hour intervals were of the same relative magnitude (Table 2). For the sixteen observations, the average net increase of phosphorus incorporation in the total phospholipids of TSH-treated slices was $99.1 \pm 7.5^4\%$ greater than the control values on the basis of initial wet weight, and $91.1 \pm 7.9^4\%$ greater on the basis of final wet weight (p values of $< .001$ for each). In eleven of twelve thyroid glands, the stimulation of phospholipid metabolism was associated with an elevation of QO_2. For the twelve experiments, average oxygen consumption was $11.5 \pm 1.6^4\%$ greater than control values in the presence of TSH, a statistically significant difference ($p < .001$). Attempts to effect a temporal dissociation between the QO_2 and the phospholipid effects were unsuccessful. The augmentation of QO_2 could be demonstrated within ten minutes following the introduction of TSH from the side-arm and remained relatively constant thereafter. When the effects of TSH upon phosphorus incorporation into thyroidal phospholipids are corrected for the concomitant increase of QO_2, the relationship between TSH and phosphorus incorporation (as judged by their partial correlation coefficient) remains statistically significant ($P < .001$).

The action of TSH *in vitro* could not be duplicated with other pituitary hormones. Incubation of sheep thyroid slices with 1.0 mg. quantities of various pituitary preparations did not augment QO_2 or phospholipids turnover to a greater extent than could be attributed to the contamination with TSH (Table 3).

Other tissues were examined for the tissue specificity of TSH (Table 4). In surviving sheep liver slices, TSH did not alter respiratory or phospholipid metabolism. These parameters were also unaffected by the addition of thyrotropin to lactating rat mammary slices, a tissue which is capable of both concentrating and organifying iodine (13).

3. *The effects of TSH upon total phospholipids and the water-soluble phosphorus of sheep thyroid slices:* In Table 5 are listed results from three experiments in which the absolute amounts of water-soluble inorganic and or-

TABLE 2. THE EFFECT OF TSH UPON THE RESPIRATORY METABOLISM AND THE INCORPORATION OF PHOSPHORUS INTO THE PHOSPHOLIPIDS OF SHEEP THYROID SLICES

Duration (hours)	Expt.* No.	Final weight (% of initial)		Control value†		+ TSH: % of control value‖	
		Control	+ TSH	P incorp. ‡ (μg./gm.)	QO_2§	P incorp.	QO_2
1	1	88.5	85.4	0.46	0.34	193.5	113.9
	2	84.8	95.9	0.54	0.40	203.7	121.3
	3	69.8	83.7	0.48	0.30	208.3	115.0
	5	77.8	67.6	1.20	0.62	164.2	100.2
Mean				0.67	0.41	192.4	112.6
2	1	88.5	85.4	0.91	0.34	234.1	113.9
	2	84.8	95.9	1.22	0.40	218.8	121.3
	4	107.5	115.5	1.57	0.42	166.2	116.4
	7	77.8	92.9	1.35	0.33	232.6	109.8
	12	78.7	76.6	0.75	0.40	185.3	105.2
Mean				1.16	0.38	207.4	113.3
3	3	69.8	83.7	1.72	0.30	221.5	115.0
	6	67.0	63.8	2.53	0.47	187.3	111.8
	11	82.0	86.0	1.14	0.36	249.1	113.8
Mean				1.80	0.38	219.3	113.5
4	8	89.7	89.2	2.62	0.37	211.1	108.9
	9	78.7	75.4	2.25	0.49	152.9	108.3
	10	70.4	81.4	2.60	0.46	208.1	113.8
	12	78.7	76.6	2.16	0.40	149.5	105.2
Mean				2.41	0.43	180.4	109.1

* Observations were obtained at two separate time intervals in experiments 1, 2, 3 and 12. Tabulated values for "final weight" and "QO_2" in these experiments represent the averages of the multiple observations.

† Absolute values for thyroid slices incubated without TSH in the suspending medium.

‡ "P incorp. (μg./gm.)": Minimal estimate of net phosphorus incorporation during the incubation period derived by dividing the observed counts in lipid extracts by the specific activity of the "phosphate media" in the side-arms. Values expressed per gm. of initial wet weight.

§ "QO_2": μl O_2 per mg. initial wet weight during the first hour of incubation.

‖ Phosphorus incorporation and respiratory activity in the presence of TSH expressed as a percentage of the control values. Added TSH consisted of 1.0 U.S.P. unit per Warburg vessel.

ganic phosphorus and lipid phosphorus of thyroid slices and the specific activities of these respective pools were measured after 4 hours of incubation. Consistent increases of specific activity by TSH could only be demonstrated for the phospholipid phosphorus. The increase of lipid specific activity could not be attributed to a commensurate reduction in the size of the thyroidal phospholipid pool. In both stimulated and in control tissues, the specific activities of the inorganic phosphorus[5] were lower than the

[5] Ennor and Rosenberg have suggested that an absolute estimate of the true inorganic phosphorus of tissues is not possible by any of the available techniques (20). The present measurements are also subject to the limitations which these authors have discussed.

TABLE 3. THE EFFECT OF PITUITARY HORMONES UPON THE RESPIRATORY METABOLISM AND THE INCORPORATION OF PHOSPHORUS INTO THE PHOSPHOLIPIDS OF SHEEP THYROID SLICES

Hormone*	Preparation	Contaminants†	Final weight (% of initial)	QO_2‡	P incorp.§ (μg./gm.)
Control	———	———	79.6	0.31	1.45
TSH	Armour #IRW	"Relatively free"	94.3	0.34	3.37
ACTH	H.B.F. Dixon	?	87.6	0.29	1.94
Prolactin	Armour #759-39CCC	?	78.7	0.32	1.70
STH	Armour #732-181	"TSH 0.05 units/mg."	80.4	0.34	2.19
FSH	Armour #264-151X	"TSH <0.1 units/mg. LH <2%"	87.3	0.30	1.71

* One mg. amounts of the varjous preparations added to each Warburg vessel. Assay of Armour TSH #IRW: "1.5–2.0 U.S.P. units per mg." All analyses performed in duplicate. Duration of incubation: two hours.

† Based on available assay figures.

‡ "QO_2": μl. O_2 per mg. initial wet weight during the first hour of incubation.

§ "P incorp. (μg./gm.)": Minimal estimate of net phosphorus incorporation into the phospholipids of thyroid slices during incubation. Values expressed per gm. of initial wet weight.

specific activities of the phosphate in the suspending medium. However, in the presence of TSH, the total tissue pool of inorganic phosphorus[5] was greater than the control values in every experiment and the organic water-soluble phosphorus was markedly increased in one of the three experiments. Specific activity relationships would indicate that the effects of TSH upon the total quantity of water-soluble phosphorus in thyroid slices cannot be entirely attributed to an increased entry of phosphate from the suspending medium.

TABLE 4. THE EFFECT OF TSH UPON THE RESPIRATORY METABOLISM AND THE INCORPORATION OF PHOSPHORUS INTO THE PHOSPHOLIPIDS OF EXTRA-THYROIDAL TISSUES *In Vitro*

Duration (hours)	Tissue	Species	Final weight (% of initial)		Control value*		+ TSH: % of control value§	
			Control	+ TSH	P incorp.† (μg/gm.)	QO_2‡	P incorp.	QO_2
2	Lactating Mammary Gland	Rat	76.4	70.0	2.23	0.77	86.1	101.3
1	Liver	Sheep	87.0	86.3	0.25	0.32	88.0	103.1

* Absolute values for control slices incubated without TSH in the suspending medium. Control and TSH media supplemented to contain 200 mg.% glucose.

† "P incorp. (μg./gm.)": Minimal estimate of net phosphorus incorporation into the phospholipids of surviving tissue slices during incubation. Values expressed per gm. of initial wet weight.

‡ "QO_2": ul O_2 per mg. initial wet weight during the first hour of incubation.

§ Phosphorus incorporation and respiratory activity expressed as a percentage of the control values. Added TSH consisted of 1.0 U.S.P. unit per Warburg vessel.

TABLE 5. THE EFFECT OF TSH ON THE PHOSPHORUS FRACTIONS OF SHEEP THYROID SLICES

Expt.* No.	TSH	Medium P†		Tissue				Phosphorus	
				Water soluble				Lipid	
				Inorganic		Organic			
		μg./ml.	S.A.‡	μg./g.§	S.A.	μg./g.	S.A.	μg./g.	S.A.
8	Control	35.2	72,003	39.6	36,731	141	39,979	247	1,145
	+ TSH	34.8	68,847	46.1	39,945	144	36,800	226	2,592
9	Control	34.6	60,640	49.6	39,716	108	40,152	214	1,131
	+ TSH	35.4	68,435	58.3	42,717	149	32,969	216	1,722
10	Control	34.4	71,433	44.9	42,680	162	27,818	223	1,257
	+ TSH	36.2	69,798	55.9	32,778	165	31,609	213	2,741

* Experimental numbers same as in Table 2.

† Final values of the suspending medium at the end of 4 hours of incubation of 280–300 mg. of sheep thyroid slices. Following tipping-in of the "phosphate-media" from the side-arms into vessels which did not contain tissue, initial P concentration was 31.0 μg./ml. with a specific activity of 100,000 counts P^{32} per minute per μg. P.

‡ S.A.: Specific activity of phosphorus adjusted to an initial value of 100,000 counts P^{32} per minute per μg. P in the "phosphate-media" of the side-arms.

§ μg./g.: Values for total "pool size" expressed on the basis of initial wet weight.

4. *Effect of TSH upon the incorporation of P^{32} into the individual phospholipids of sheep thyroid slices:* By fractionating lipid extracts into individual components, it could be demonstrated that the effects of TSH *in vitro* are not distributed over the entire phospholipid population. Results of a typical experiment are presented in Table 6 and summary data from seven observations in six experiments are tabulated in Table 7. Although the values for the individual phospholipids are expressed in terms of specific activities, differences in absolute radioactivity were of the same order of magnitude.

TABLE 6. THE EFFECT OF TSH UPON THE INCORPORATION OF PHOSPHORUS INTO THE INDIVIDUAL PHOSPHOLIPIDS OF SHEEP THYROID SLICES
Expt. No. 11*

	Specific activity, $\frac{\text{counts } P^{32}\dagger}{\mu\text{g. P}}$	
	Control	+ TSH
Phospholipid type:		
Phosphatidylcholine	835	944
Phosphatidylethanolamine	574	980
Phosphatidylserine	922	3,024
Phosphoinositide	2946	10,857
Phosphatidic Acid	655	1,205

* Sheep thyroid slices (300 mg. initial wet weight) incubated for 3 hours with 25 μc.$NAH_2P^{32}O_4$. Added TSH consisted of 1.0 U.S.P. unit.

† Specific activity expressed as counts per minute P^{32} per μg.P and corrected to an initial specific activity of 100,000 counts per minute per μg.P in the "phosphate media" in the side-arms. Individual phospholipids resolved by two-dimensional paper chromatography following mild alkaline hydrolysis of lipid extracts.

TABLE 7. THE EFFECT OF TSH UPON THE INCORPORATION OF PHOSPHORUS INTO THE INDIVIDUAL PHOSPHOLIPIDS OF SHEEP THYROID SLICES

Experiment No.*	2	3	6	11	12		13
Duration (hours)	2	3	3	3	2	4	4
	% of control specific activity†						
Phospholipid type:‡							
Phosphatidylcholine	97	127	83	113	104	117	86
Phosphatidylethanolamine	473	317	495	171	159	185	251
Phosphatidylserine	—	—	—	328	287	254	—
Phosphoinositide	835	410	256	369	337	190	161
Phosphatidic Acid	—	—	—	184	134	228	—

* Experimental numbers same as in Table 2.

† Individual phospholipids in lipid extracts resolved by two-dimensional paper chromatography following mild alkaline hydrolysis. Specific activities of individual components in TSH-treated slices expressed as a percentage of the specific activity of the individual phospholipids in control tissues.

‡ Analyses for phosphatidylserine and phosphatidic acid were not performed in Experiments 2, 3, 6 and 13.

In contradistinction to the *in vivo* findings (v.s.), the action of TSH *in vitro* was principally upon phosphatidylethanolamine, phosphatidylserine and phosphoinositide. Thyroidal phosphatidylcholine was minimally, if at all, affected during 2 to 4 hours of incubation with TSH. By virtue of this heterogeneity, the stimulatory effects of TSH upon individual phosphoglycerides were frequently greater than the apparent differences in unfractionated lipid extracts.

DISCUSSION

In the intact sheep, the acute administration of TSH effects an increased incorporation of P^{32} into every thyroidal phosphoglyceride and water-soluble intermediate of phosphoglyceride metabolism. Although of interest, such *in vivo* findings must be interpreted with caution. Simple increase of thyroidal blood flow could result in an increased delivery of radioactive label and so elevate the specific activity of every thyroidal phosphorus-containing component. Thus, *in vitro* systems, wherein vascular factors may be discounted, are superior for differentiating between primary and secondary events in trophic hormone action. However, such endeavors are usually challenged on the grounds that metabolic pathways may not be similar *in vivo* and *in vitro*. This objection does not appear to obtain for thyroidal phospholipids. In a previous report, the phospholipid metabolism of unstimulated thyroid tissue was examined *in vitro* (7). In the present studies, the *in vitro* observations were documented *in vivo*. It was shown that *in vivo*, as *in vitro*, the individual phosphoglycerides exhibit different specific activities and the thyroidal phosphoinositide is more heavily labelled during the early incorporation of P^{32} than any other lipid component. Moreover, it was demonstrated that *in vivo*, as *in vitro*, the water-soluble phosphomonoesters, phosphorylcholine and phosphorylethanolamine,

satisfy the radioactive criteria for precursors of phospholipid phosphorus, whereas the phosphodiesters, glycerylphosphorylcholine and glycerylphosphorylethanolamine, behave like products of phospholipid catabolism.

In view of the qualitative parallelisms in the *in vivo* and the *in vitro* metabolism of the phospholipids of unstimulated thyroid tissue, it was gratifying to be able to confirm with sheep thyroid slices the findings which Morton and Schwartz first reported with beef thyroid tissue (10). TSH *in vitro* uniformly increased the incorporation of P^{32} into the total thyroidal phospholipids. The phenomenon could not be duplicated with extrathyroidal tissues nor with other pituitary preparations. TSH also elevated thyroidal QO_2 as others have reported (21–23); however, the *in vitro* respiratory effects were neither as constant nor as great as the changes in phospholipid metabolism.

From the available data, one cannot say whether the enhanced turnover of phospholipid phosphorus constitutes the primary site of TSH action. However, it may be inferred that this represents one of the earliest *in vitro* manifestations of thyrotropic stimulation. In the present studies, the net incorporation of phosphorus into thyroidal lipids was uniformly doubled by TSH. The relative differences between stimulated and control tissues were as great after one as after four hours of incubation. In view of the linearity of the resting phosphorus incorporation, the data may be extrapolated to suggest that the phospholipid effect occurs almost immediately. Comparable differences of phosphorus incorporation into thyroidal phosphorus fractions (such as the water-soluble phosphate esters, for example) were not observed. However, phosphorus fractions constitute complex mixtures; hence, the data do not preclude the possibility of marked differences in the labelling of individual components of the fractions. Indeed, on the basis of the marked enhancement of total lipid radioactivity by TSH, one might predict early effects upon the specific activities of selected, water-soluble phosphoglyceride precursors. The dangers inherent in attempts to derive absolute information from the mean specific activity of complex mixtures are best exemplified by the studies in which thyroidal lipid extracts were resolved into the individual phosphoglycerides. This disclosed that the stimulation of phosphorus incorporation by TSH was not distributed over the entire phospholipid population. The apparent two-fold increase in the labelling of the total unfractionated phospholipids was shown to have resulted from a) two to eight-fold increases in the net incorporation of phosphorus into the cephalins and the phosphoinositide, and b) minor to negligible changes in the net phosphorus incorporation into the component which accounts for half (7) of the thyroidal lipid phosphorus —i.e. phosphatidylcholine (lecithin).

In view of the adequate synthesis of lecithin in the unstimulated thyroid slice (7), it is difficult to interpret the failure of TSH *in vitro* to augment the turnover of lecithin phosphorus as much as that of the

phosphorus in the other phosphoglycerides. A lack of cytidinetriphosphate (24) or D-α, β-diglycerides (24) seems unlikely since these factors are equally necessary for biosynthesis of the TSH-sensitive phosphatidylethanolamine and the TSH-insensitive phosphatidylcholine. A deficiency of specific precursors such as physphorylcholine (24) does not appear to be rate-limiting since abundant stores of labelled phosphorylcholine can be demonstrated in aqueous extracts of TSH-treated slices. Thus, the minimal action of TSH upon thyroidal lecithin during four hours of incubation may reflect an absolute or a relative unresponsiveness of such enzymes as phosphorylcholine-cytidyl transferase (24) or phosphorylcholine-glyceride transferase (24), a phenomenon which might have been masked by the more rapidly progressing events *in vivo*. Resolution of the diverse possibilities must await future investigation. However, the present evidence for a selective action of TSH upon thyroidal phospholipids *in vitro* would not be consonant with a generalized effect upon communal energy stores (such as ATP) as the basis for TSH action. Rather, the selectivity would suggest that TSH acts by a more specific reorientation or realignment of intrathyroidal metabolic pathways.

What are the physiological ends which this reorientation subserves? For the moment, precise physiological meaning cannot be assigned. In other systems, phospholipids have been implicated in ion transports (25) and in the maintenance of the structural organization of multi-enzyme complexes (26). Perhaps the observations of the Hokins afford the most interesting speculative possibilities. Hokin and Hokin, and Hokin and Sherwin have shown that the *in vitro* stimulation of protein secretion by the addition of cholinergic agents (27, 28) or pancreozymin (29) to slices of pancreas, or by the addition of either adrenergic or cholinergic agents to slices of salivary gland (30) is uniformly accompanied by an increased turnover of phospholipid phosphorus. On the basis of this evidence, Hokin and Hokin have postulated that phosphate turnover in phospholipids plays a functional role in protein secretion (29, 30). If their concepts may be extrapolated to thyroid tissue, then it is possible that the *in vitro* effects of TSH upon thyroidal phospholipids may be linked to the activation of glandular secretion. The latter would be in keeping with the suggestion of Keating, Rawson, Peacock and Evans (31) that the primary locus of TSH action is upon thyroidal secretion, and would afford a biochemical foundation for the histological observations of Dempsey (32) and De Robertis (33) that an increase of intrathyroidal sudanophilic materials and colloid droplets are among the earliest manifestations of TSH action.

SUMMARY

Pathways of thyroidal phospholipid metabolism have been examined in intact sheep and earlier *in vitro* findings have been confirmed. Acute administration of pituitary thyrotropin (TSH) resulted in an increased in-

corporation of radioactive phosphorus *in vivo* into all of the phospholipids and water-soluble phospholipid intermediates of sheep thyroid tissue.

During incubation of surviving sheep thyroid slices, the addition of TSH to the suspending medium effected a prompt increase in the incorporation of radioactive phosphorus into thyroidal phospholipids, and a less pronounced, but significant, elevation of thyroidal oxygen consumption. The actions of TSH could not be duplicated in extrathyroidal tissues nor with other pituitary hormones.

Fractionation of lipid extracts disclosed that the stimulation of phospholipid metabolism *in vitro* was not distributed over the entire phospholipid population. Although TSH enhanced the turnover of phosphorus in thyroidal cephalins and phosphoinositide, the major thyroidal phospholipid component, phosphatidylcholine, was minimally affected during four hours of incubation.

The stimulation by TSH of phospholipid metabolism and the selective effects upon a limited number of thyroidal phosphoglycerides *in vitro* are discussed with reference to the mechanism of action of TSH.

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METABOLITES OF RADIOACTIVE L-THYROXINE AND L-TRIIODOTHYRONINE[1]

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L-THYROXINE and L-triiodothyronine are subject to transformation in the animal by several different enzyme systems. The most active deiodinating system for these substances is found in the liver; but the kidney shows some deiodinating activity (1). Kidney slices can deiodinate thyroxine (T4[2]) to triiodothyronine (T3[2]) by a deiodinating system which is conditioned by the level of T4 available in the tissues (2). An enzyme system which conjugates glucuronide is present in the liver, and in much smaller amounts, also in the kidney. It can transfer glucuronic acid to various phenols (3, 4), including thyroxine (5). This system probably is responsible for the synthesis of thyroxine glucuronide by the perfused liver (6) and also for the synthesis of triiodothyronine glucuronide. (7). Oxidative deamination of T4 and T3 by other enzyme systems could lead to the formation of tetraiodothyropyruvic acid and triiodothyropyruvic acid, and these substances have been found in the bile and urine of the rat (8). Oxidative decarboxylation of these pyruvic acid derivatives of T4 and T3 would lead to the formation of tetraiodothyroacetic acid (Tetrac[2]) and triiodothyroacetic acid (Triac[2]); the latter has been found in kidney and muscle (9, 10) after administration of T3. Tomita, Lardy, Larson and Albright have demonstrated the enzymatic conversion of T4 to Tetrac and of T3 to Triac by the mitochondrial fraction of the kidney of the rat (11). It is not known whether any of these chemical changes of thyroxine or triiodothyronine are essential for the biologic activity of these substances, and information is scarce concerning the relative importance of the different enzyme systems in the metabolism of thyroxine and triiodothyronine in the whole animal.

We have shown previously that the metabolism of thyroxine or triiodothyronine is less complete in the absence of the liver and that urinary excretion of inorganic iodide is decreased and that of organic iodine com-

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[2] In this paper the following abbreviations have been used: T4 for thyroxine; T3 for triiodothyronine; Tetrac for tetraiodothyroacetic acid and Triac for triiodothyroacetic acid.

pounds is increased (12). These urinary metabolites of thyroxine and triiodothyronine have been studied further by column and paper chromatography and by paper electrophoresis. In addition, the metabolites in plasma or serum have been studied in the presence and absence of the liver and kidneys.

METHODS

Dogs weighing 6 to 24 kg. were used in this study. Total hepatectomy was performed on 24 dogs, usually from 6 to 12 months after cellophane bands had been placed on the portal vein and abdominal vena cava by the technic of Grindlay and Mann (13). Bilateral nephrectomy was performed on nine of these dogs at the time of hepatectomy, and on five control dogs. In some of the dogs complete thyroidectomy was performed. Only the two or three parathyroid glands embedded in the lobes of the thyroid were removed.

Strong iodine solution (Lugol's solution) was given to most of the dogs 18 hours before operation. Within an hour after operation T4 or T3 labeled with radioiodine (I^{131})[3] was injected intravenously into the dogs in doses from 0.8 to 7.0 μg. per kilogram of body weight with specific activities from 5 to 55 microcuries per microgram. Glucose dissolved in physiologic saline solution was given by continuous intravenous injection to all the dehepatized dogs at the rate of 150 mg. per kilogram per hour for 6 to 15 hours and then at higher rates, as indicated, up to 350 mg. per kilogram per hour.

Similar amounts of glucose were given to the dehepatized-nephrectomized dogs but in smaller volumes of saline solution. Saline injections were given to the nephrectomized dogs.

Urine was collected from the dehepatized dog by an indwelling catheter throughout the experiment, and blood was collected at intervals. The radioactivity of urine and plasma (or serum) was measured in a well-type NaI (thallium-activated) scintillation counter. The total amount of I^{131} circulating in the plasma was calculated on the assumption that 4.5% of the body weight of the dog was plasma. The compounds of I^{131} in urine and blood were separated by three different methods: column chromatography, paper chromatography and paper electrophoresis.

Column Chromatography.—Samples of 0.5 to 1 ml. of urine or plasma were pipetted directly onto a kieselguhr column, and the I^{131} compounds were eluted first with 4:1 butanol-chloroform saturated with 0.5N NaOH, then with 9:1 butanol-propanol also saturated with 0.5N NaOH, and finally with water as previously described (14). The radioactivity of the effluent from the column was recorded continuously, and the effluent was collected in test tubes by a fraction collector. The radioactivity in the individual collecting tubes was measured in the well-type scintillation counter and the total radioactivity in each column peak was calculated.

Paper Chromatography.—Protein and lipide-free extracts of plasma were made with acid-butanol, followed by the addition of chloroform, and extraction into 2N NH_4OH as previously described (15).

Aliquots of plasma extracts or untreated urine were applied at the lower right-hand corner of 10 by 12-inch sheets of Whatman 3 MM filter paper. Two-dimensional chromatograms were developed in butanol-dioxane-2N NH_4OH, overnight, and then in tertiary amyl alcohol-2N NH_4OH for 2 days. Nonradioactive markers of iodide (I), T4,[4] T3,[4]

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[3] Obtained from Abbott Laboratories.

[4] We are grateful to Smith, Klein and French for gifts of L-thyroxine, L-triiodothyronine and L-triiodothyroacetic acid, and to Dr. Rosalind Pitt-Rivers for triiodothyroacetic acid and the L-tetraiodothyroacetic acid used in these experiments.

Tetrac[4] and Triac[4] were frequently run with the plasma and urine. Radioautographs of the chromatograms were made on "no-screen" x-ray film. The thyronines and thyroacetic acids were located on the chromatograms by the diazo reaction (16) and the iodide with palladous chloride (17).

Column peaks were often rechromatographed on paper. The effluent from the column was dried under reduced pressure at 30° C in the presence of small amounts of thiouracil (18), then dissolved in 50% ethanol and pipetted onto the filter paper. Some of the peaks of the conjugate were incubated with β-glucuronidase according to the method of Taurog, Briggs and Chaikoff (19), and the hydrolytic products chromatographed on paper.

Paper Electrophoresis.—Aliquots of plasma extracts or concentrates of column peaks were applied to strips of Whatman 3 MM filter paper measuring 30 by 3 cm. and electrophoretic separation was carried out for 15 hours in barbital buffer at pH 8.6 with an ionic strength of 0.075, with a current of 0.62 ma. in the Spinco electrophoretic apparatus, Model R, Series C. Nonradioactive markers of T4, T3, Tetrac and Triac were often run with the specimens.

RESULTS

Thyroxine Metabolites. Urine.—Six radioactive peaks were found in column chromatograms of urine collected during the first 6 hours after administration of radioactive T4 to dehepatized dogs (Fig. 1). These peaks contained T4, two unidentified iodine compounds in peaks 2 and 3, iodide, the conjugate and a small residual fraction. Chromatograms of urine collected at later intervals showed a small forepeak, and much less resolution between the T4 peak and peak 2, a large conjugate peak and a smaller iodide peak. The conjugate peak was often asymmetric and sometimes showed a double peak suggesting that more than one substance might be present. The peaks of radioactivity found in the column effluent as illustrated in Figure 1 and the percentage of the radioactivity in each are listed in Table 1.

Two-dimensional paper chromatograms of these samples of urine showed definite radioactive spots for iodide and the conjugate (Fig. 2). There was also a radioactive spot to the right of iodide (I). No radioactivity was found at the sites of the nonradioactive markers of T3 and Tetrac.

When column peak 2 was studied by paper electrophoresis, some radioactivity was found at the origin indicating the presence of some T4 but most of the I^{131} had migrated 5 and 10 cm. (Fig. 3). When column peak 3 was studied, the electrophoretic pattern showed little radioactivity at the origin and radioactive bands at 5 and 8 cm.

When the conjugate peak from the column was studied by paper chromatography, the I^{131} moved almost entirely to the conjugate region. When the conjugate peak was incubated with β-glucuronidase and the products were separated by paper chromatography, two radioactive spots were found, one in the T4 region and another just to the right which would correspond to the position of the I^{131} compound in peak 2 of the column (Fig. 4). These two spots run so closely together on a two-dimensional chromatogram that they would appear as one substance on a one-dimensional chromatogram.

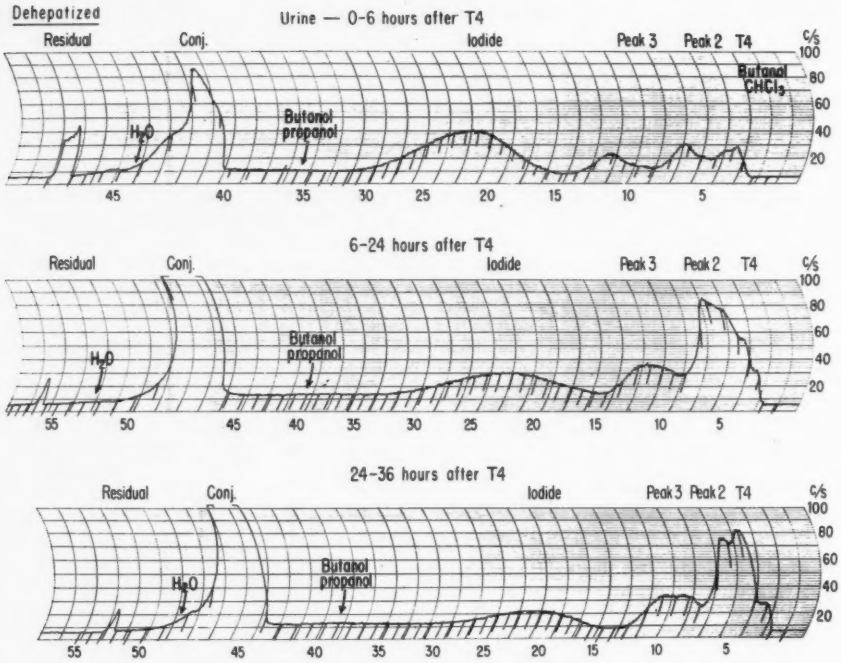

FIG. 1. Column chromatograms of urine after the injection of T4 to a dehepatized dog. The records moved from right to left at the rate of one division per hour. The solvent changes are indicated by the arrows. The tube changes are indicated by the lines pointing downward from the curves. The number of tubes, each containing 7.5 ml., which were collected, are indicated on the base line. The radioactivity is indicated on the ordinate in counts per second.

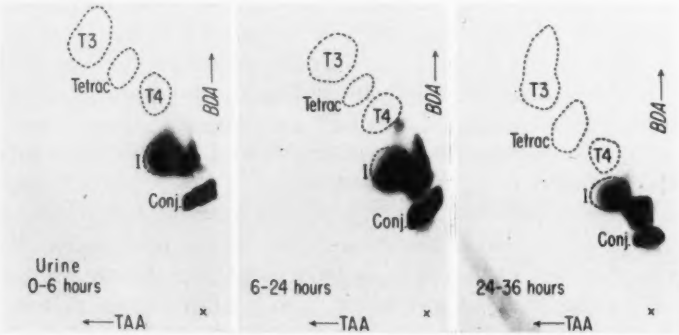

FIG. 2. Paper chromatograms of untreated urine. The solvent mixtures used were butanol-dioxane-2N NH_4OH (BDA) and tertiary-amyl alcohol-2N NH_4OH (TAA). The location of the nonradioactive markers are indicated by the dotted lines.

TABLE 1. METABOLITES OF T4 IN DEHEPATIZED DOGS

Dog	Hours after T4	Total I^{131} % of dose (cumulative)	I^{131} distribution, % in each area						
			T4	Peak 2	Peak 3	I^-	Conj.	Dity.*	Residual
			Urine						
1	0–6	2.9	36.7	5.4	4.4	25.1	15.0	6.8	6.5
	6–12	7.1	13.3	10.9	10.6	19.3	43.0		3.8
2	0–6	10.1	14.2	12.4	25.1	10.2	32.1	2.8	3.3
	6–12	18.0	24.6	8.8	14.7	33.3	12.0		6.5
3	0–6	4.6	8.7	13.6	28.3	21.9	21.5		5.9
	6–20	14.2	4.4	18.3	30.8	22.0	20.7		3.9
4†	0–6	3.9	23.6	5.8	5.9	29.2	30.7		4.7
	6–21	12.4	16.0	17.9	15.8	27.7	21.6		1.0
5	0–6	2.8	6.9	8.0	7.4	45.5	25.7		6.3
	6–24	18.9	27.8		11.0	28.3	31.2		1.7
	24–30	23.6	27.0		9.8	22.5	39.1		1.6
			Serum						
3	6	34.1‡	63.3		20.8	10.4	3.7		1.8
	20	17.6	59.8		23.7	10.6	2.6		3.2
4	6	23.2							
	21	19.5	61.1		26.0	5.5	4.2		3.2
5	6	28.0							
	24	16.2	61.1		26.6	4.4	4.6		3.2
	30	14.2	51.4		37.0	2.5	5.3		3.5

* Diiodotyrosine.
† Thyroidectomy in dog 4 also.
‡ Calculated on basis that the plasma volume is 4.5% of the body weight.

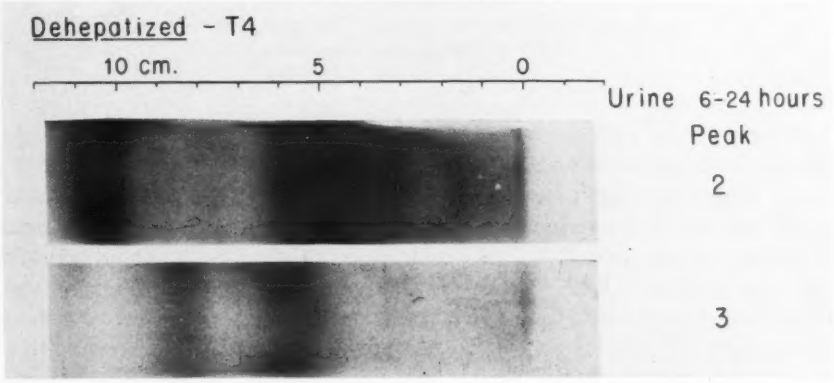

FIG. 3. Peaks from column chromatograms of urine from Figure 1 studied by paper electrophoresis. *6 to 24 hours.* Peak 2, tubes 5 to 9; peak 3, tubes 11 to 13.

When the conjugate was incubated with the buffer only, most of it was unchanged, but a small amount moved to a position just below the T4 spot. When the conjugate peak from bile was examined in a similar manner, the same two spots appeared after incubation with β-glucuronidase, but most of the radioactivity was found in the T4 spot (20).

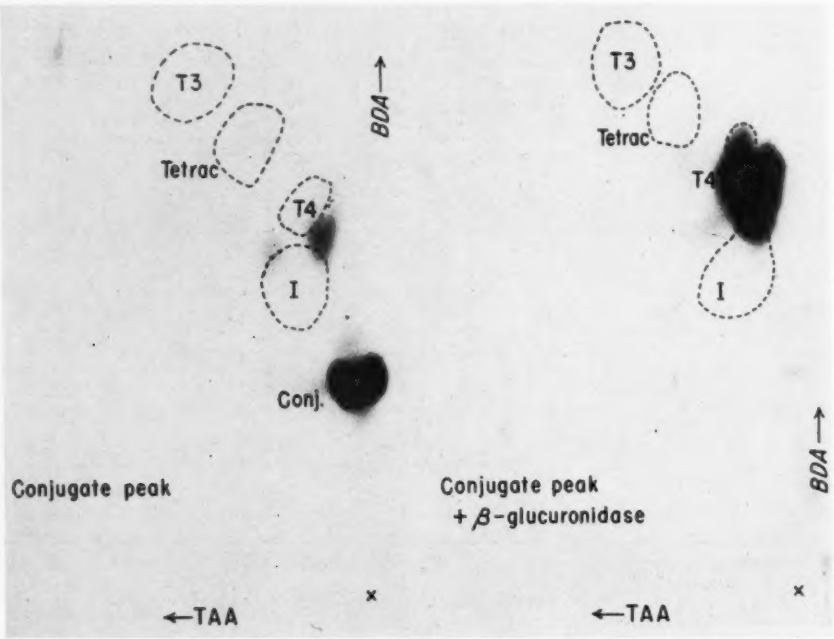

FIG. 4. Paper chromatograms of the conjugate peak from the column chromatogram of urine of a dehepatized, thyroidectomized dog. The urine was collected from 6 to 21 hours after injection of T4. *Left.* Conjugate after incubation with the buffer only. *Right.* The effect of incubation with β-glucuronidase in the buffer.

Blood.—Relatively little free T4 was excreted in the urine by the dehepatized dog, and an appreciable amount was still circulating in the blood 24 and 36 hours after its administration. Column chromatograms of serum showed a sharp peak of T4 merging into peak 2 with a prolonged downward slope, little iodide, a small conjugate peak and a small residual fraction (Table 1). When the T4 peak was examined by paper chromatography, a large spot was found in the T4 region, and there were two spots to the right of this, a conjugate spot and a small spot of Tetrac. When peak 2 was examined in a similar manner, most of the radioactivity appeared in the spot to the right of iodide but some also appeared as T4 and the conjugate. When the T4 peak was studied by paper electrophoresis, a dark band of radioactivity was found at the origin indicating the presence

of T4 (10). A light band of radioactivity was found in the Tetrac position at about 7 cm., and another heavy band was found at 10 cm. Peak 2 produced bands at approximately 5 and 11 cm.

When urinary excretion of I^{131} was eliminated in the dehepatized dog by bilateral nephrectomy, more of the metabolites of T4 remained in the

TABLE 2. METABOLITES OF T4 IN PLASMA

Dog	Hours after T4	Total I^{131}, % of dose	I^{131} distribution, % in each area					
			T4	Peak 2	Peak 3	I^-	Conj.	Residual
Dehepatized, nephrectomized dogs								
6	6	18.3	80.3			6.3	11.4	1.9
7	0.3	80.1	63.1	10.5	8.4	14.9	2.0	1.1
	24	16.1	37.2	34.0		11.6	14.0	3.2
8	6	29.8*	60.1		24.6	5.7	8.9	0.7
	24	17.9*	20.4		50.6	4.6	20.8	3.6
9	6	23.4	78.0	8.6	1.7	4.5	4.5	2.7
	12	16.8	60.3	16.7	5.1	7.5	8.5	2.1
	24	15.0	28.4	35.2	11.0	4.0	16.8	4.6
	37	15.1	9.8	32.3	31.7	2.9	18.2	5.1
10	6	22.6	90.2		3.0		3.1	3.7
	24	15.3	53.4		15.9	11.1	14.8	4.8
	39	12.6	24.7	27.7	17.3	3.2	19.5	7.5
Nephrectomized dogs								
11	6	24.9	79.7		4.2	13.5	1.9	0.6
	24	7.0	29.3	5.7	4.0	50.5	3.8	6.7
	36	4.4	16.1	2.3	0.8	76.7	2.3	1.8
12†	6	24.4	63.2		8.4	16.3	7.7	4.4
	12	18.3	37.8		16.5	29.1	11.5	5.0
	24	15.1	11.2		23.1	47.6	12.8	5.3

* Serum.
† Biliary obstruction also.

plasma. At 6 hours after injection most of the I^{131} in the blood was present as T4 (Table 2). At later intervals peaks 2 and 3 were clearly visible in column chromatograms of plasma, and there was a large conjugate peak. In contrast, column chromatograms of plasma from a nephrectomized dog with intact liver 36 hours after injection of T4 showed peaks of T4 and inorganic iodide only.

Paper chromatograms of serum extract of dehepatized, nephrectomized dogs after suitable extraction showed a large T4 spot in the 6-hour serum and a much smaller amount of T4 at 24 hours. There were prominent spots corresponding to peaks 2 and 3 of urine and also of the conjugate.

Triiodothyronine Metabolites. Urine.—Column chromatograms of urine of dehepatized dogs after the injection of T3 showed radioactive peaks a

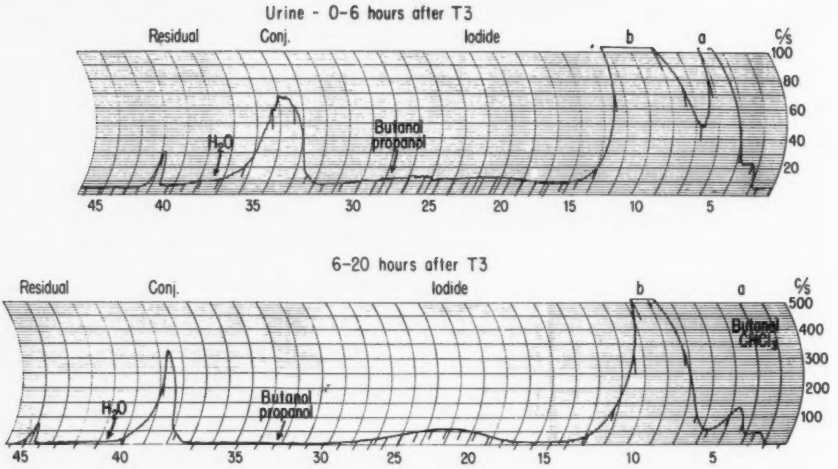

FIG. 5. Column chromatograms of urine from a dehepatized thyroidectomized dog after injection of T3. a and b are unidentified compounds. The total amount of I^{131} excreted in the urine was 5.4% of the dose at 6 hours and 48.0% of the dose at 20 hours.

and b, iodide, conjugate and a small amount of the column residual fraction (Fig. 5). Peak a was higher in early samples of urine than in later samples. Peak b contained most of the radioactivity (Table 3).

Paper electrophoretic patterns of the urine of dehepatized dogs showed that T3 was present although its presence was masked on the column chromatograms by peaks a and b (Fig. 6). A prominent radioactive band was found at the same distance as the nonradioactive marker of Triac in the patterns from peaks a, b and the conjugate. However, Triac was not found on paper chromatograms of these samples. Other radioactive bands were found, but it is difficult to interpret their significance.

Paper chromatograms of untreated urine from a dehepatized dog after injection of T3 showed the presence of T3 in early samples of urine and also the presence of iodide and two spots, a and b, which appeared between T4 and I^- but were not always completely resolved (Fig. 7).

TABLE 3. METABOLITES OF T3 IN URINE OF DEHEPATIZED DOGS

Dog	Hours after T3	Total I^{131}, % of dose (cumulative)	I^{131} distribution, % in each area						
			T4	a	T3	b	I^-	Conj.	Residual
13	0–6	5.4		23.0		58.0	6.0	11.5	1.5
	6–20	48.0		7.3		69.5	11.4	8.9	2.8
14	0–22	5.8				58.7	11.9	25.9	3.5
15	0–6	8.8	1.3	12.0	64.8	5.5	1.2	12.3	2.8
	6–23	35.7		9.8		61.4	12.8	14.5	1.4

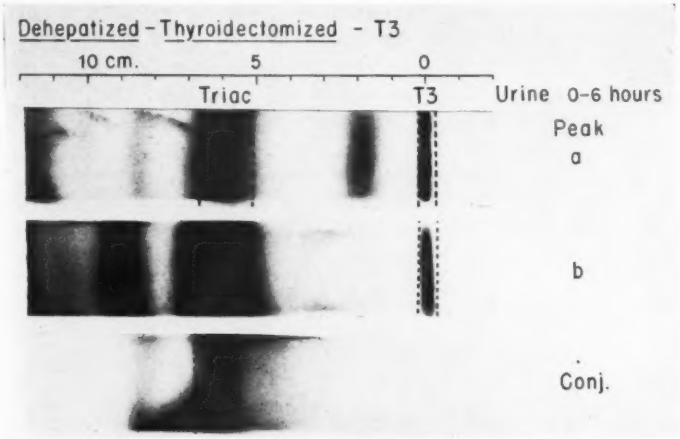

FIG. 6. Peaks from column chromatograms of urine from figure 5 studied by paper electrophoresis. The position of the nonradioactive markers of T3 and Triac are indicated. *0 to 6 hours*. Peak a, tubes 5 to 7; peak b, tubes 9 to 14; conj. (conjugate), tubes 34 to 37.

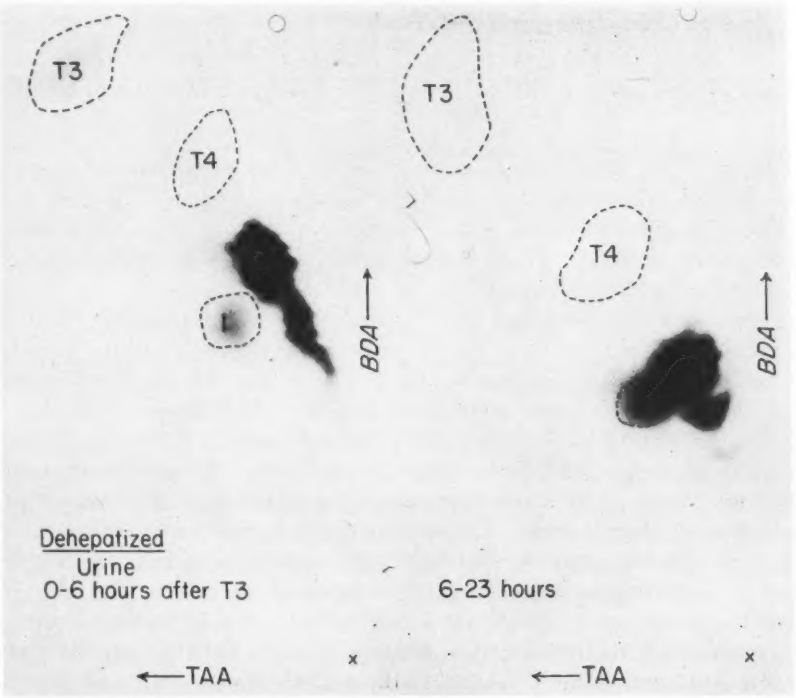

FIG. 7. Paper chromatogram of urine of a dehepatized dog after injection of T3. The total amount of I^{131} excreted at 6 hours was 8.8% of the dose and at 23 hours 35.7% of the dose.

TABLE 4. METABOLITES OF T3 IN PLASMA

Dog	Hours after T3	Total I^{131}, % of dose (cumulative)	I^{131} distribution, % in each area						
			T4	a	T3	b	I^-	Conj.	Residual
Dehepatized, nephrectomized dogs									
16	1	16.5	3.6		90.9		0.6	2.2	2.7
	6	12.8	2.6	34.3		54.1	2.0	3.1	3.9
	12.5	16.8	2.7	35.1		62.6	1.1	5.7	2.7
17	6	18.0	1.5	11.6		76.9	0.9	1.0	8.1
	22	28.7	1.3	11.8		72.9	1.1		12.9
18*	1	20.3	2.6	6.9	74.8	9.4	0.9	2.0	3.3
	6	22.1	2.2	13.5		75.1	1.4	3.9	3.7
	24	30.6	2.1	8.4		76.2	0.8	12.4	
19	12	19.2	1.4	11.3		76.3	1.4	5.2	4.4
	24	20.0	2.3	11.1		75.0	2.3	6.3	2.9
Nephrectomized dogs									
20	1	16.2	1.8		59.6	14.1	12.9	9.2	2.4
21	6	6.6	0.8		22.0		70.9	1.6	4.7
	36	5.8			7.7		89.0	1.0	2.3
22	6	6.2	1.8		16.5	15.0	59.4	1.1	6.2
	36	4.9			7.6		84.1	5.2	2.9

* Serum.

Blood.—Column chromatograms of serum from a dehepatized dog after injection of T3 showed the presence of peaks a and b with a peak of T3 between, and a small peak of the conjugate at 6 hours. Peaks a, b and T3 were poorly resolved. At 20 hours the early peaks were still unresolved, and they merged with iodide.

Much better resolution of the I^{131} compounds was obtained in the serum of the dehepatized dog by paper chromatography. One hour after injection of T3, a large spot of T3 was found, and spots a and b appeared between the T4 and iodide spots, often close together. At later intervals the T3 spot became small and spots a and b became larger.

When urinary elimination of the metabolites of T3 was prevented by bilateral nephrectomy on the dehepatized dog, these metabolites with the exception of the conjugate accumulated in larger amounts in the blood. At 1 hour after injection of T3 a large T3 peak was found in serum but even then it was merging into the neighboring peaks a and b (Fig. 8). At 6 hours peaks a and b completely hid any T3 that might still be present. There was little further change in the appearance of the column chromatograms of plasma after 6 hours. Most of the radioactivity was found in peak b (Table 4). The I^{131} in the plasma of the nephrectomized dog with intact liver at 6 hours after T3 was injected was present almost entirely as

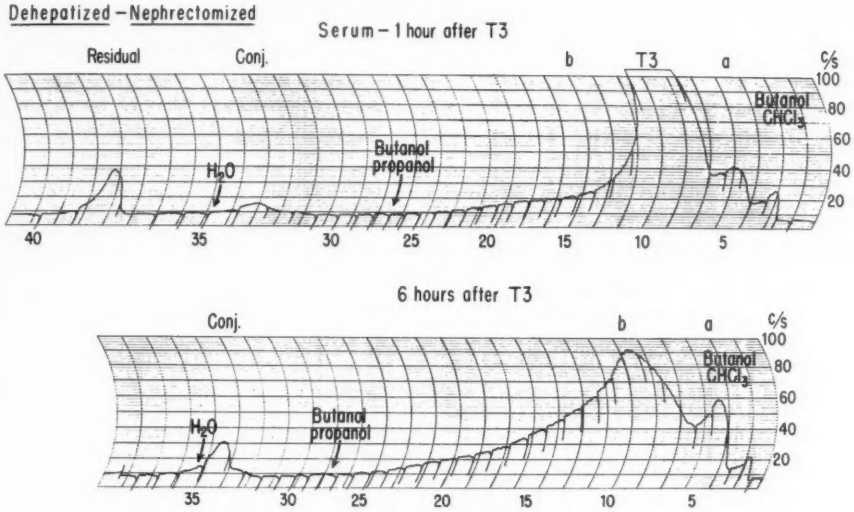

FIG. 8. Column chromatograms of serum from a dehepatized, nephrectomized dog after the injection of T3. The total I^{131} circulating in the plasma at 1 hour was 20.3% of the dose and at 6 hours 22.1% of the dose.

inorganic iodide, but there was also a trace of T3. At later intervals only iodide was found.

When column peaks of plasma or serum were studied by paper electrophoresis, a large amount of T3 was found in the 1-hour serum but little was found at later intervals. Both compounds a and b migrated in the electrical field.

Paper chromatograms of extracts of serum from dehepatized, nephrectomized dogs showed a large spot of T3 at 1 hour after injection and only a trace at 6 hours. Compounds a and b appeared between the T4 and iodide regions and were often poorly resolved. A small amount of conjugate was seen.

COMMENT

In early studies of the basal metabolic rate following removal of the liver, Mann and Boothby (21) demonstrated that the liver did not have any direct control over the basal production of heat. The metabolic rate and the respiratory quotient were unaltered by hepatectomy, and the administration of glucose increased the respiratory quotient as it does in the intact animal. This suggests that the thyroid hormone does have peripheral action in the dehepatized dog. Likewise, peripheral degradation of both T4 and T3 occurs in the dehepatized dog but with less formation of inorganic iodide than in the normal dog.

A striking similarity was apparent between the column chromatograms

of urine excreted by the dehepatized dog during the first 6 hours after injection of T4 and the chromatograms of urine excreted by the dog with biliary obstruction. Even the conjugate peak was markedly elevated in both of these dogs. The peaks of the organic iodine compounds were higher than in the urine of the normal dog. In the urine of the dehepatized dog the amount of conjugate excreted continued to increase with time, and the amount of inorganic iodide formed decreased. T4 was excreted as the glucuronide, and one of the derivatives of T4 was excreted in this form also. Roche, Michel, Etling and Nunez (22) have found that 3:3′:5′ triiodothyronine is conjugated about as well as T4 and to a much greater extent than 3′:3:5 triiodothyronine while scarcely any conjugate of 3:3′ diiodothyronine occurs in the bile. They considered that the amount of conjugation is probably regulated by the degree of dissociation of the phenolic group of the iodothyronines. Information is not available as to whether alterations in the alanine side chain of the iodothyronines affects the conjugation. Our studies show that conjugation of T4 is not limited to the liver nor does it require the presence of the kidneys.

The combined use of column and paper chromatography and paper electrophoresis for the study of the metabolites of T4 and T3 leaves many unsolved problems. However, this study has delineated the characteristics of the two unidentified derivatives of T4 and also has shown that these compounds occur in blood and urine in much greater amounts than the better known derivatives, such as T3 and Tetrac. Isolation and identification of these substances, therefore, would be desirable as this might throw some additional light on the metabolism of T4. Little T3 was found in the dehepatized dogs whether thyroidectomized or not.

Much less conjugate was excreted by the dehepatized dog after the injection of T3 than after injection of T4, and little, if any, accumulated in the blood of the dehepatized, nephrectomized dog. The lack of accumulation of the conjugate of T3 in the blood of such dogs may indicate that the kidney does play a significant role in its formation. Compounds a and b need identification as they occur in large amounts and may be more important than the better known derivative, Triac. Although compounds a and b appear in the same general region on both column and paper chromatograms as the unidentified metabolites of T4, they are probably not common derivatives of both T4 and T3.

SUMMARY

The major metabolites of thyroxine and triiodothyronine which occur in blood and urine have been studied by three technics, namely, column chromatography, two-dimensional chromatography and paper electrophoresis. These metabolites are found in much larger amounts in the urine of the dehepatized dog than in the urine of the normal dog and also are

found in large amounts in the plasma, particularly, in the dehepatized, nephrectomized dog.

The major metabolites of thyroxine include the glucuroconjugate of thyroxine and two unidentified iodine compounds, one of which was also present in the conjugated form. Triiodothyronine was rarely seen on paper chromatograms and then only in small amounts. Small amounts of tetraiodothyroacetic acid, which is not separated from thyroxine by column chromatography, were found on paper chromatograms of serum.

The major metabolites of triiodothyronine included two unidentified compounds and a smaller amount of conjugated triiodothyronine. Radioactivity was found in the region of triiodothyroacetic acid in paper electrophoretic patterns but none was found in that region on paper chromatograms of urine or serum.

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NOTES AND COMMENTS

EFFECTS OF RELAXIN ON UTERINE WEIGHT OF IMMATURE RATS

Considerable research has been done on the effects of relaxin on the pubic symphysis (1, 2, 3), bovine cervix (4), and contractility patterns of the uterus (5), but very little work has been done on its overall effects on endocrine organs. These experiments were done to determine the effects produced by subcutaneous injections of 1 mg./day of relaxin[1] into immature rats for a period of one week. The adrenal glands of males and

TABLE 1. EFFECT OF AGE ON THE WEIGHT OF THE UTERUS OF INTACT RATS GIVEN DAILY INJECTIONS OF 1 MGM. OF RELAXIN

Age at necropsy	No. of animals		Avg. body weight in gm.		Wet ut. weight in mg.		Mg. % wet ut. wt.	
	Con.	Inj.	Con.	Inj.	Con.	Inj.	Con.	Inj.
21	14	—	33	—	21	—	62	—
28	18	20	62	67	39	52	63	78
35	21	22	83	97	58	85	69	88
42	10	15	97	102	79	141	81	138
49	10	11	116	132	146	215	126	163

Age at necropsy	Dry ut. weight in mg.		Mg. % dry ut. wt.		% increase in dry wt.	% H_2O	
	Con.	Inj.	Con.	Inj.		Con.	Inj.
21	3.3	—	9.9	—	—	84.4	—
28	6.2	7.6	10.0	11.4	22.6%	84.1	85.4
35	9.1	15.9	11.0	16.4	71.4	84.3	81.3
42	13.1	23.1	14.5	22.6	76.3	83.4	83.6
49	23.7	35.5	20.4	26.9	50.2	83.8	83.5

females; the seminal vesicles, ventral prostates, and testes of males; and the ovaries of females showed no marked change in weight on this regimen. However, the uteri showed a marked hypertrophy over non-treated controls, and vaginal opening was noted in some treated animals as early as 33 days of age (normal average: 42–45 days), although this premature opening was highly variable. The effect of age on uterine hypertrophy was then studied, with uterine weights and body weights obtained from untreated 21, 28, 35, 42 and 49 day old female albino rats and from animals treated for one week with 1 mg./day of relaxin and killed at 28, 35, 42 and 49 days of age (Table 1). Uterine weights of the injected animals were consistently greater than the controls, with the greatest relative increase in the animals killed at 42 days of age. A slight increase in body weight by the injected animals was noted. There is no effect on uterine water concentration.

In view of this effect, and knowing that estrogen injection increases uterine weight, experiments were performed to determine if the relaxin were acting as a pituitary stimulant to FSH production, as a gonadotropin, as a simple direct stimulant of the uterus, or as a stimulant acting synergistically with estrogen. To this end a group of young fe-

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[1] Relaxin furnished by the Princeton Laboratories, Princeton, New Jersey.

male rats was ovariectomized and different doses of estrogen were injected for one week to determine the amount required to sustain the uteri at weights equal to those of controls of the same age. It was found that 0.025 μg. of estradiol-17β achieved this effect. Six groups of rats were begun on treatment at 28 days of age (Table 2). Group A was ovariectomized and served as a control; Group B was ovariectomized and received 1 mg. of relaxin daily. The uterine weights of the rats of these two groups were similar, 6.5 and 6.6 mg. % dry weight. Group C was ovariectomized, received 0.025 μg. of estradiol per day, and at the end of the period showed uteri close to those of the normal nontreated animals (Group D), with mg. % dry weights being 10.7 and 10.1 respectively. The largest uterine weights, again in close agreement, were displayed by Group E which was ovariectomized and received both the relaxin and the estrogen, and Group F, the

TABLE 2. UTERINE WEIGHTS OF INTACT AND OF GONADECTOMIZED RATS GIVEN DAILY INJECTIONS OF 0.025 μg. OF ESTRADIOL-17β AND 1 MG. OF RELAXIN

Group	No. of animals	Treatment		Uterus avg. wet wt.	S.E.	Avg. body weight	Mg. % wet wt.	Mg. % dry wt.
		Receiving relaxin	Receiving estrogen					
A	6 spayed females	−	−	24.4 ±	2.2	77.3	31.5	6.5
B	6 " "	+	−	23.0 ±	3.7	86.1	26.7	6.6
C	5 spayed females	−	+	38.0 ±	3.9	68.4	55.6	10.7
D	8 intact females	−	−	57.5 ±	9.3	90.6	63.5	10.1
E	5 spayed females	+	+	73.3 ±	9.3	90.2	81.3	19.2
F	18 intact females	+	−	76.4 ±	9.0	94.5	78.9	16.4

normal animals receiving relaxin. Mg. % dry weights were 19.2 and 16.4 respectively.

Thus as relaxin has no effect on the uteri of ovariectomized rats receiving no estrogen, it cannot act by itself. The similarity between Groups C and D showed the dosage of estrogen to be essentially correct. Because Groups E and F so closely approximate each other, with uteri significantly above normal, relaxin must act synergistically with estrogen, and as it can produce this effect in the absence of ovaries, it does not act as a gonadotropin.

SUMMARY

Evidence is offered indicating that (a) injections of relaxin into young intact rats cause uterine hypertrophy, and (b) relaxin synergizes with estradiol-17β and thereby effects larger uterine weights in ovariectomized rats.

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[2] Studies carried out during the tenure of a Lederle Medical Faculty Award.

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THE ABILITY OF 17-ETHYL-19-NORTESTOSTERONE TO BLOCK ETHIONINE-INDUCED FATTY LIVER IN RATS

In 1955 Farber and Segaloff (1) demonstrated that androgens inhibited the fatty infiltration of the livers of rats treated with ethionine. These workers observed that even a relatively weak androgen such as androstenediol provided as much protection to the liver as did the more potent testosterone propionate. They suggested that this

TABLE 1. THE EFFECT OF 17-ETHYL-19-NORTESTOSTERONE, TESTOSTERONE PROPIONATE AND PROGESTERONE ON ORGAN WEIGHTS AND TOTAL LIVER LIPIDS OF ETHIONINE TREATED CASTRATED RATS

Group	Compound	Dose	Body wt.	Liver wt.	Seminal vesicle wt.	Liver total lipid
		mg./kg.	gm.	gm.	mg.	%
1	control	0	239	9.31	37.5 ± 5.63*	9.24 ± 0.91*
2	control (without ethionine)	0	281	8.41	34.5 ± 1.70	2.19 ± 0.06‡
3	17-ethyl-19-nortestosterone	0.1	251	9.24	34.5 ± 3.42	8.39 ± 1.18
4	17-ethyl-19-nortestosterone	0.3	278	10.4	39.4 ± 1.66	5.32 ± 1.18†
5	17-ethyl-19-nortestosterone	0.5	252	9.82	32.4 ± 2.79	2.62 ± 0.65‡
6	17-ethyl-19-nortestosterone	5.0	258	9.25	113 ± 11.3‡	4.11 ± 0.46‡
7	testosterone propionate	0.04	268	10.9	41.0 ± 5.42	15.1 ± 0.62‡
8	testosterone propionate	0.08	274	10.1	51.2 ± 2.58†	3.19 ± 0.30‡
9	testosterone propionate	0.2	262	10.4	100 ± 6.00‡	4.41 ± 0.57‡
10	testosterone propionate	2.0	275	10.2	309 ± 7.08‡	4.76 ± 0.61‡
11	progesterone	4.0	347	10.2	31.7 ± 1.83	11.9 ± 1.08

* Standard error of the mean.
† $P < .05$.
‡ $P < .01$; unmarked values are not significantly different from control.

might be a function of the anabolic properties of the hormone. More recently Saunders and Drill (2) described several testosterone derivatives in which a separation of anabolic and androgenic properties had been achieved. In addition these workers have shown that these substances have progesterone-like actions (3). It has been possible for us to confirm the effectiveness of such an anabolic agent in blocking the fatty liver due to ethionine administration and also to show that neither the androgenic nor the progestational properties of such a testosterone derivative participate in inhibiting the lipid mobilizing response of the animal to ethionine.

METHODS

Adult male rats (Sprague-Dawley) which had been castrated 4 weeks previously were divided into groups of 6 animals. Each group was treated for 8 days with a synthetic steroid administered intramuscularly. On the 8th day all animals were fasted and at 2 hour intervals were given aqueous dl-ethionine intraperitoneally in 3 equal doses that totaled 1 mg. per gm. body weight. On the 9th day the animals were sacrificed and their livers and seminal vesicles were removed and the livers were quick-frozen and stored. The initial analyses for total lipid in the liver samples were carried out on the petroleum ether soluble material extracted from

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the tissue by alcohol-ether (4). Most tissue extracts were prepared by the equivalent but more convenient chloroform-methanol extraction procedure of Folch *et al.* (5). The total lipid in all extracts was analyzed by the colorimetric oxidative method of Bragdon (6).

RESULTS

A comparison of 17-ethyl-19-nortestosterone and testosterone propionate in terms of the ability of these compounds to inhibit fatty infiltration of the liver after ethionine treatment is given in Table 1. These data indicate that, although 0.5 mg./kg. of 17-ethyl-19-nortestosterone was not androgenic as shown by lack of seminal vesicle growth, this dose completely inhibited lipid infiltration of the liver. Testosterone propionate, on the other hand, seemed not to protect the liver except at androgenic doses (0.08 mg./kg. or greater).

Progesterone was ineffective in preventing fatty livers whereas complete protection was provided by 17-ethyl-19-nortestosterone. The doses used were approximately equivalent in terms of the progestational activities of these compounds (3). It is evident, therefore, that it is not this property of the testosterone derivative that blocked the appearance of the ethionine fatty liver syndrome.

SUMMARY AND CONCLUSION

It is apparent that the lipid infiltration of the liver caused by ethionine treatment of the castrate rat can be effectively blocked by certain testosterone derivatives as exemplified by 17-ethyl-19-nortestosterone at a dose that does not stimulate the growth of secondary sex organs. The protective action of such a substance does not depend on progesterone-like action but upon a property apparently associated with its myotrophic activity. This lends further support to the hypothesis that the major defect imposed by ethionine administration is one in protein metabolism secondary to which may be the alteration in lipid metabolism that has been studied here.

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BODY WEIGHT CHANGES IN HYPOPHYSECTOMIZED RATS FOLLOWING IMPLANTATION OF SOMATOTROPIN PELLETS

During the course of preliminary experiments (1) designed to show the direct action of bovine growth hormone, (somatotropin, STH) on the mammary gland, it was found that hypophysectomized rats showed good body weight increases in response to sub-

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cutaneously-implanted STH pellets. The lyophilized STH could be compressed in a pellet-making apparatus and then heated in a dry air oven for 1 hour at 100° C without loss of potency.

EXPERIMENTAL

In order to learn more about the growth response of hypophysectomized rats to STH pellets, parallel series have been run in which amounts of STH equal to those contained in 4 different sized pellets have been given by the usual subcutaneous injection technic over a period of 5 weeks. Long-Evans male rats were hypophysectomized at 40 days of age and treatment was begun immediately in the form of daily injections or weekly pellet implantations below the dorsal skin. The purified STH was mixed with equal parts of cholesterol in order to increase the bulk of the pellet and facilitate the handling of fragments cut to equal the following weights of STH:0.7, 1.4, 2.8 mg. In order to control any change that might have occurred in the STH during compression and mixing with cholesterol, the STH given in daily injections was prepared by pulverizing the cholesterol-STH pellets and suspending the material in water. The daily injections corresponding to the weekly implantations of the 3 pellet sizes mentioned were in the amounts of 0.1, 0.2 and 0.4 mg. respectively. Cholesterol was not added in making the 14 mg. pellet and the corresponding group of rats in this case received daily injections of 2.0 mg. of the hormone.

When tested in standard hypophysectomized assay rats, the STH in a daily dose of 10 μg. was found to stimulate tibial epiphyseal cartilage growth. The same test animals showed no adrenocortical or thyroid stimulation after 7 daily doses of 2 mg. Ten normal controls and 9 untreated hypophysectomized controls were also studied during the treatment period of 5 weeks and the post-treatment period of 7 weeks. After 5 weeks the normal rats were hypophysectomized and their weight decrement curves compared with those of the experimental groups in their post-injection period. Body weights were charted at weekly intervals over the 12-week period after which time necropsies were performed and hypophysectomies were checked for completeness.

RESULTS

The results are shown in Figure 1 and Table 1. By day 14, no statistically significant differences ($P > 0.05$) could be detected between the average weight increments of corresponding implanted and injected groups (4 and 7, 5 and 8, 6 and 9). By this time the 9 rats implanted with 14.0 mg. pellets had shown an average gain of 60.6 ± 4.7 gm., while the corresponding 10 rats receiving 2.0 mg. injections daily had gained 72.0 ± 2.6 gm. ($P < .05$ but $> .02$). The growth curve of the group injected with 2.0 mg. STH daily followed almost precisely that of 10 normal males of the same age. The 9 hypophysectomized controls had lost an average of 7.8 ± 2.8 gm.

After the second week and following the third implantation of pellets the weight curves of the corresponding implant and injection groups began to diverge; and in each case the injected groups showed higher average gains for the 5 week period. The significance of these differences on day 35 may be judged by the following P values: groups 4 and 7, $P < .01$; groups 5 and 8, $P < .001$; groups 6 and 9, $P > 0.2$, but $< .05$; groups 12 and 13, $P < .001$. The rats receiving 2.0 mg. STH daily by injection had continued to match the normals. The hypophysectomized controls had remained constant.

Because of the decreasing effectiveness of all pellets implanted weekly, a second set of 10 rats received implants from the same batch of 0.7 mg. STH pellets every 4 days. The growth curve of this group matched that drawn for group 5, injected with 0.2 mg. daily. The STH in a pellet of this size dissolves slowly and usually is absorbed in 4–5 days; whereas the large pellets acquire fibrous capsules, that permit residues to persist for 3 weeks or more.

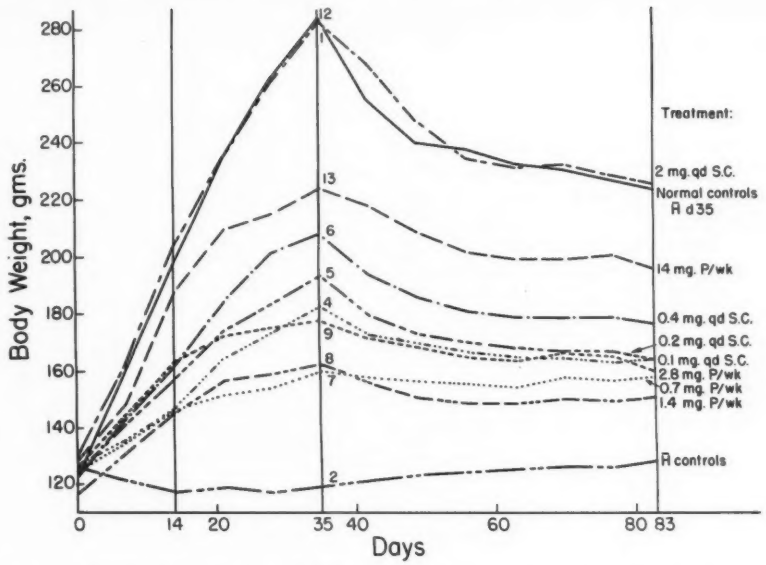

FIG. 1. Weight increment and decrement curves of hypophysectomized rats implanted weekly with STH pellets (P/wk) or injected daily (q.d.s.c.) for 5 weeks as listed.

After the administration of STH was stopped in all groups, the body weights decreased rapidly at first and then remained constant after day 60 in most animals until the end of the observation period. The weight decrement curve for the normal group hypophysectomized on day 35 was strikingly similar to that of the hypophysectomized group 12, injected daily for 34 days with 2.0 mg. STH and then followed until day 83 without treatment. In general the weight decrements in the various groups were proportional to the peak weights attained during treatment. The rats of group 13 implanted with the 14.0 mg. pellets showed a significantly smaller weight loss and decrement rate than did those injected with 2.0 mg. daily (Group 12) ($P < .01$). The difference in the over-all weight gains in these 2 groups was significant, with a P value of <0.05 but >0.02. There were no significant differences in the final weights of the 3 groups on

TABLE 1. BODY WEIGHT CHANGES IN NORMAL AND HYPOPHYSECTOMIZED RATS DURING AND AFTER A REGIMEN OF STH INJECTED DAILY OR IMPLANTED WEEKLY AS PELLETS

Gr.	No. rats	Init. av. body wt.	Mg. STH		Mean body wt. change and S.E.M.			Final av. body wt.	Final body wt. change
			Daily weekly inj.	pellet	Day 0–14	Day 0–35	Day 35–83		
1	10*	123	—	—	+74.3±3.8	+160.4±7.2	−59.4±2.9	224	+101 ±5.5
2	9	126	—	—	− 7.8±2.8	− 6.7±1.9	+ 9.0±2.7	128	+ 2.3±0.8
4	10	127	0.1	—	+20.4±2.1	+ 56.6±4.6	−19.6±3.6	164	+ 37.0±3.6
5	10	124	0.2	—	+33.3±3.0	+ 70.4±5.1	−29.6±2.9	164	+ 40.8±3.9
6	10	123	0.4	—	+37.5±3.7	+ 85.7±5.8	−30.9±3.7	177	+ 54.8±6.0
7	8	125	—	0.7	+21.4±3.9	+ 34.7±4.7	− 2.2±2.9	158	+ 32.5±3.9
8	6	116	—	1.4	+29.6±1.6	+ 47.3±1.9	−12.5±2.3	151	+ 34.8±1.8
9	7	126	—	2.8	+35.4±3.6	+ 50.6±8.5	−16.7±5.5	160	+ 33.9±5.6
12	10	130	2.0	—	+72.9±2.6	+148.4±4.9	−52.4±4.9	226	+ 96.5±6.0
13	9	128	—	14.0	+60.6±4.7	+ 95.4±8.2	−26.7±4.9	197	+ 68.7±8.7

* Hypophysectomized on day 35 of experiment; all others hypophysectomized on day 0 = 40 days of age.

smaller pellets (7, 8 and 9) and the 2 groups on the lower injected doses (4, 5). Group 6, injected with 0.4 mg. STH daily showed a slightly better over-all gain than the 5 groups just mentioned (P values <0.05 but >0.02).

SUMMARY

For 2 weeks the growth curve of a group of normal male rats was closely imitated in hypophysectomized males injected daily with 2.0 mg. systemic doses of STH or an over-all equivalent weight of this hormone in the form of a 14.0 mg. pellet implanted subcutaneously, each week. During the same period, weekly implantation of STH pellets weighing 0.7, 1.4 and 2.8 mg. induced the same growth response respectively as did 0.1, 0.2, and 0.4 mg. of STH given in daily injections. During the following 3 weeks the 4 groups of rats receiving the daily injections showed better growth rates than did the corresponding dose groups implanted with pellets.

After treatment with STH for 5 weeks, the weight decrement of all rats was charted for 7 weeks. All groups gradually lost weight for about 3 weeks after treatment; the weights then remained essentially constant for the last 4 weeks. The weight decrement curve of the group on 14.0 mg. pellets was not so steep as that of the 2.0 mg. daily injected group, suggesting that the encapsulated pellets probably released small residues of hormone during this phase of the experiment.

The weight decrement curve of the normal group of rats hypophysectomized at the fifth week was quite similar to that of the 2.0 mg. daily injected group after their 5 weeks' treatment was stopped.

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PUBLIC HEALTH SERVICE

Readers of ENDOCRINOLOGY may be interested in a program to improve the quality of laboratory mice obtainable commercially. A set of *Minimum Standards for the Commercial Production of Randombred and Inbred Laboratory Mice* has been prepared.

Copies of the *Minimum Standards* may be obtained free of charge from Mr. Berton F. Hill, Executive Secretary, Institute of Laboratory Animal Resources, National Academy of Sciences, 2101 Constitution Avenue, N.W., Washington 25, D.C., or from Dr. George L. Wolff, National Institute of Health, Section on Screening, Cancer Chemotherapy, National Service Center, Bethesda 14, Md.

BOOK REVIEW

THE ADRENAL CORTEX, by I. Chester Jones. Cambridge, at the University Press, pp. 316, 9 plates and 35 text-figures, 1957. Price, $7.00.

In view of the fact that the greater part of our knowledge concerning the adrenal cortex is derived from observations made in a very few mammalian species, it is gratifying to read of the attempts that have been made to extend our knowledge of this gland so that it encompasses a wide variety of vertebrates. In writing this book the author has set himself the task of rendering a coordinated account of what is known of the structure and function of the adrenal cortex in the various classes of vertebrates; he has, in my opinion, succeeded in making a unique and valuable contribution, no easy task today in view of the immense literature extant on this gland. The opening chapter is concerned with the adrenals in Eutherian mammals; subsequent chapters deal with these glands in fish, amphibians, reptiles, birds and the more primitive mammals, the monotremes and marsupials. The final chapters include a discussion of the comparative embryology of the vertebrate adrenal gland and an attempt to correlate glandular structure and function. The bibliography, containing over one thousand items, is a most comprehensive one and enhances, in no small measure, the usefulness of this monograph. Although the documentation is, in places, a bit overwhelming, it is to Dr. Chester Jones' credit that almost never does he content himself with a mere recital of the pertinent findings but usually attempts a critical review of the data under discussion.

The opening chapter, dealing as it does with the adrenals in Eutherian mammals, is perforce the longest. It includes information relating to the gross and microscopic anatomy of the adrenal cortices in these species, the biochemistry of the adrenal steroids, control of adrenocortical secretion, functions of the adrenal cortex, relationship of the cortex to peripheral tissue, adrenal-gonad relationships and a brief description of some natural abnormalities of function. To cover such ground in 1957 necessarily means repeating much of what has already been said. The virtue of this presentation lies in the range of information subsumed by a single, well-written chapter. It could be recommended without qualification as an excellent introduction to the subject were it not for a disconcerting aspect of this chapter and that is the rather abrupt transitions in the nature of the audience that the author chooses to address. Almost alongside such patently preceptive statements as, "The pituitary can be removed surgically from an animal (Smith, 1927). This operation is referred to as hypophysectomy, and means that the anterior, intermediate and posterior lobes of the pituitary have been removed.", one encounters quite sophisticated discussions of some of the knotiest problems presently confronting the specialist in this area. The only serious factual errors that could be detected in this chapter pertain to the chemistry of the steroids and they are few in number. They are particularly unfortunate in view of Dr. Chester Jones' earnest attempts to introduce the tyro to the nomenclature of steroid structure; he has provided a valuable glossary containing definitions not usually available without recourse to somewhat esoteric chemical publications.

The subsequent chapters on the lower vertebrates provide a variety of insights. They indicate that, in many of the vertebrate classes, the present state of knowledge concerning the adrenal cortex is analogous to what we knew of the mammalian adrenal cortex in the late Nineteen Twenties or early Nineteen Thirties. The very brevity of these chapters clearly depicts how woefully little we know about adrenocortical function in most vertebrates. These chapters also reveal that much of what work has been done on

the adrenals of lower vertebrates has been strongly influenced by our findings in mammals; many of these experiments have been devised and the results interpreted in the light of our knowledge of adrenocortical function in rats and dogs. Surely this need not necessarily be a valid frame of reference. Perhaps future investigators will display greater independence. In any event, if any further stimulus be needed to learn more about the adrenals of lower vertebrates, it should be provided by the author's most able recapitulation of the current situation.

At this point the reviewer would like to insert a personal opinion about terminology. Throughout the book Dr. Chester Jones makes repeated reference to "salt-electrolytes," in allusion, of course, to the principal inorganic ions of biological fluids such as sodium and potassium. It is my feeling that this awkward, misbegotten phrase deserves, and indeed is long overdue for, a quiet burial.

The chapter on the embryology of the vertebrate adrenal, while short, is nevertheless an authoritative one and displays to good advantage the author's not inconsiderable talent for teaching with the written word. The concluding chapter, an attempt to correlate structure and function, takes up the proposed theories pertaining to the sites of origin and possible migratory activities of adrenocortical cells within the cortex itself, and also those theories that relate specific hormonal secretions to the various zones of the cortex. It has the strongest subjective flavor of any chapter in the book and is properly provocative.

The plates are made up of microscopic sections that have been chosen well and that have been, in general, reproduced with quite good fidelity of detail. Judicious selection is likewise evident in the text-figures. The diagrams, however, are not exceptional. One in particular, that portraying the possible pathways for the biogenesis of adrenocortical steroids, is so complicated that it most probably will slow rather than facilitate the learning process.

At first glance, it might seem that this book would be primarily of interest to the zoologist. That may be so, and yet I believe that it can be read with some advantage by all who have an interest in the adrenal cortex. Perhaps it will encourage investigators to exercise greater freedom in the choice of experimental animals. Customarily, we give but little thought to the selection of the species best suited for the solution of a particular problem; almost reflexly we order rats or dogs. If nothing else, it is the convenient thing to do. Our persistent adherence to this practice leaves us today in the position of being able to lay defensible claim to only a most circumscribed knowledge of many aspects of adrenocortical function.

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